DIFFERENTIAL EXPRESSION OF THE GMCSF GENE IN THE IMMUNE SYSTEM IS REGULATED BY EPIGENETIC FACTORS

A thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

by

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DECLARATIONS

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgment is made in the text.

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. Animal ethics approval was granted by the University of Tasmania Animal Ethics Committee.

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At the time of writing, the following publication has arisen from data presented in this thesis:

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ABBREVIATIONS

Ab	antibody
Ac	acetylation
AP-1	activator protein 1
APC	antigen presenting cell
Asf1	antisilencing function 1
ATP	adenosine triphosphate
aza or azacytidine	5-aza-2-deoxycytidine
BAF	Brg1/Brm associated factor
BCR	B cell receptor
BL	baseline time point
bp	base pair
Brg1	Brahma-related gene 1
Brm	Brahma
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CBP	CREB binding protein
CD	cluster of differentiation
CD28RE	CD28 response element
CD28RR	CD28 response region
CD28RRm	CD28RR mutant
cDNA	complementary DNA
CHART-PCR	chromatin accessibility by real-time PCR
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CK-1	cytokine-1 element
CLE0	conserved lymphokine element 0
CO_2	carbon dioxide
CpG	5' CG 3'
CRC	chromatin remodelling complex
CsA	Cyclosporin A
CTCF	CCCTC-binding factor
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNMT	DNA methyltransferase
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid

EZH2	enhancer of Zeste homolog 2
FCS	foetal calf serum
g	gram
g	relative centrifugal field
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCN5	general control nonderepressible 5
GM0.2	pXP1-mGM0.2 plasmid
GM-CSF	granulocyte-macrophage colony stimulating factor
H2A	histone H2A
H2B	histone H2B
H3	histone H3
H4	histone H4
HAT	histone acetyltransferases
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGM	human GM-CSF
HMG	high mobility group
HMT	histone methyltransferase
I	calcium ionophore
IFN	interferon
Ig	immunoglobulin
Igf2	insulin-like growth factor 2
IKK	IκB kinase
IL	interleukin
IP_3	inositol-1,4,5-trisphosphate
IS	immunological synapse
IκB	inhibitor of NF-κB
κB	NF-κB binding site in GM-CSF promoter
K	lysine
kb	kilobase
kDA	kilodalton
M	molar
MBD	methyl binding domain
me	methylation
MECP2	methyl CpG binding protein 2
mg	milligram
mGM	mouse GM-CSF
MHC	major histocompatibility complex
mL	millilitre
mM	millimolar
MNase	micrococcal nuclease

mRNA	messenger RNA
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometre
nM	nanomolar
nmol	nanomole
NS	nonstimulated
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHO5	acid phosphatase
PHO8	alkaline phosphatase
PI	PMA/ionophore
PKC	protein kinase C
PMA	phorbol myristate acetate
PolII	RNA polymerase II
PRC2	Polycomb repressive complex 2
qPCR	quantitative PCR
RHD	Rel homology domain
RNA	ribonucleic acid
RNase	ribonuclease
RS	restimulated time point
RT-qPCR	reverse transcription qPCR
RUNX	runt-related
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sp1	specificity protein 1
Sp1m	Sp1 binding site mutant
Spt	suppressor of Ty
SR	stimulus removed time point
SWI/SNF	switch/sucrose nonfermentable
T/P	TSA/PI treated time point
TCR	T cell receptor
TE	Tris/EDTA buffer
TF	transcription factor
Th	T helper cell
Thn	naïve helper T cell
TI	total input
TNF	tumour necrosis factor
TNT	Tris/NaCl/Tween buffer
Tris	Tris(hydroxymethyl)aminomethane
TSA	Trichostatin A

TSS	transcription start site
U	unit
V	volt
WT	wild type
µF	microfarad
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar

ABSTRACT

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine that stimulates the production of leukocytes as part of an immune response. The role that GM-CSF plays in the immune system is reliant on its tightly controlled expression, both temporally and spatially. The overall aim of this thesis was to investigate the factors that contribute to the correct temporal and spatial expression of *GM-CSF* in immune cells.

It was found that while *GM-CSF* expression can be stimulated in murine T cells but not B cells, key transcription factors involved in *GM-CSF* gene expression were present in both cell types, leading to the hypothesis that epigenetic mechanisms underlie this differential response. In support of this, differences in DNA methylation, histone modifications and the presence of chromatin remodelling proteins were detected at the *GM-CSF* promoter between the two cell types.

DNA methylation levels were higher at a CpG dinucleotide in the *GM-CSF* promoter in T compared to B cell lines, and DNA methylation of the *GM-CSF* promoter blocked expression from a reporter plasmid. Demethylation of the promoter was not sufficient to enable *GM-CSF* gene expression in B cells, although it increased its expression in T cells. The effect of removing the CpG dinucleotide, which is contained in an Sp1 transcription factor binding site, was also examined. In a transiently transfected reporter model, removal of the Sp1 site resulted in loss of

promoter activity. However, in a stably integrated transgene model, the Sp1 mutant promoter exhibited an increased response to stimulation. The differential response of the promoter mutant between the transient and stably transfected models suggests that the chromatin environment of the gene plays an important role in transcriptional regulation.

To further examine the importance of chromatin in *GM-CSF* gene regulation, histone modifications were examined at the *GM-CSF* promoter in T and B cell lines. Several key differences were observed. In T cells, acetylation of histone H3 was increased at the *GM-CSF* promoter relative to B cells. Increasing promoter acetylation levels by treatment with the histone deacetylase inhibitor Trichostatin A (TSA) facilitated expression of *GM-CSF* in the B cell lines in response to stimulation. Furthermore, TSA treatment in combination with DNA demethylation had a synergistic effect on *GM-CSF* expression in both T and B cells. In contrast to histone acetylation, histone H3 lysine 27 trimethylation was lower at the *GM-CSF* promoter in T cells relative to B cells. Finally, the chromatin remodelling protein Brg1, which is known to interact with acetylated histones, was present at the *GM-CSF* promoter in T cells at higher levels than in B cells.

These data suggest that enrichment of histone H3 acetylation and Brg1 and decreased H3K27Me3 contribute to the establishment of a 'permissive' chromatin environment at the *GM-CSF* promoter in T cells, which is not present at the promoter in B cells. A 'permissive' chromatin environment can be established at the *GM-CSF* promoter

in B cells following treatment with TSA, which increases histone acetylation. This allows remodelling of the promoter chromatin and subsequent gene expression in response to immune signals. However, this induced 'permissive' state is not maintained. Following removal of the inducing stimulus in A20 B cells, the chromatin is reset to its original 'repressive' state and the *GM-CSF* gene becomes unresponsive to subsequent stimulation.

Chapter One – Introduction

1.1 – The immune system

The mammalian immune system is a complex network that has evolved to protect the host against pathogenic organisms, toxins, and cancerous cells. Broadly speaking, the immune system can be divided into two facets: the innate and the adaptive immune systems (reviewed in Chaplin, 2010). Innate immunity includes physical barriers to pathogen entry as well as cells capable of activating inflammation, providing a "front-line" rapid, but non-specific, response to pathogenic insult. Adaptive immunity, on the other hand, has the capability to recognise and target specific pathogenic molecules, but responds at a slower rate than the innate immune system. These two systems thus work in synergy to control pathogenic invasion.

The innate immune system broadly recognises structures such as bacterial cell wall components and viral nucleic acids, and activates an inflammatory response. Mast cells respond to the presence of pathogens (and also tissue damage) by releasing an array of factors such as histamine, tumour necrosis factor alpha (TNF-α) and many other signalling molecules, cytokines and chemokines. These cause vasodilation and extravasation of fluids, as well as orchestrating the activation and recruitment of other immune cells such as neutrophils (Nathan, 2002). Phagocytosis of pathogens and infected cells by macrophages and dendritic cells leads to antigen presentation on the cell surface, which activates components of the adaptive immune system. Briefly, antigen-presenting cells (APCs) display antigen on their cell surface bound

to the major histocompatibility complex (MHC) proteins. Professional APCs, such as dendritic cells, will phagocytose exogenous pathogens and express the antigen bound to MHC II, while infected cells will display antigen on their surface bound to MHC I. These MHC/antigen complexes are then recognised by lymphocytes. MHC I complexes are recognised by $CD8^+$ "cytotoxic" T cells, triggering clonal expansion of these cells to recognise and destroy all cells presenting this specific antigen complex. MHC II complexes, on the other hand, are bound by $CD4^+$ "helper" T cells (Th), which become activated and differentiate into Th1, Th2 or Th17 cells. These then produce distinct sets of cytokines that can activate other immune cells, both innate and adaptive, and modulate the response (reviewed in Medzhitov, 2007).

Clearly, an effective immune response relies heavily on the ability of cells to rapidly produce the appropriate molecules, such as cytokines, in response to an activating stimulus, such as MHC/antigen binding. Equally importantly, this activation must be restricted to the correct cell type at the correct time, as aberrant expression of cytokine genes can lead to chronic inflammation and autoimmune disease. Overexpression of cytokines has been implicated in the pathology of several autoimmune conditions. Examples include interleukin-32 (IL-32) and rheumatoid arthritis (Shoda *et al*, 2007), IL-25 and asthma (Angkasekwinai *et al*, 2007), and IL-10 and macrophage-mediated demyelinating polyneuropathy (Dace *et al*, 2009). Therefore, the mechanisms regulating the activation of cytokine genes in immune cells are of great interest.

1.1.1 - T cell activation and signalling pathways

For correct activation of T cells to occur, two signals must be received by the cell (Bretscher, 1999). The T cell receptor (TCR) complex, along with the co-receptors CD4 or CD8 (depending on T cell subtype), binds the MHC/antigen complex presented on the surface of an APC. Specifically, the TCR complex binds the antigen while CD4/CD8 bind the MHC, with CD8 binding MHC I and CD4 binding MHC II (reviewed in van der Merwe and Davis, 2003). The second major signal involved, without which T cells fail to activate properly and become anergic, is binding of a costimulatory receptor, such as CD28, inducible T cell costimulatory (ICOS), or cytotoxic T-lymphocyte antigen 4 (CTLA4) (reviewed in Rudd and Schneider, 2003). These molecules are expressed on the surface of T cells, and can be bound by ligands present on the surface of an APC. For example, CD28 is bound by the ligands B7-1 and B7-2, which are expressed on the surface of dendritic cells and B cells in response to antigen (Mondino and Jenkins, 1994). The requirement for the cooperative action of two signals ensures that T cells are only activated upon contact with an appropriately activated APC, preventing autoimmune activation.

Binding of the TCR complex and costimulatory receptor by their ligands triggers the activation of intracellular signalling cascades, ultimately resulting in the activation of a variety of transcription factors and their translocation to the nucleus, where they act to regulate the expression of target genes. Briefly, when the TCR complex binds presented antigen and CD4/CD8 binds the MHC, they form a cluster in close

physical proximity on the cell surface, known as an immunological synapse (IS). The cytoplasmic tail of CD4/CD8 is associated with a tyrosine kinase, Lck, which upon formation of the IS phosphorylates tyrosine residues in the CD3 and ζ-chain subunits of the TCR complex. This initiates a signalling cascade that activates several different signalling pathways resulting in activation of transcription factors, such as (nuclear factor of activated T cell) NFAT and Rel/NF-κB family members, in the nucleus (reviewed in Kane and Weiss, 2003; Weil and Israël, 2006). Activation of phospholipid Cγ1 (PLCγ1) by the signalling cascade leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor, causing a release of intracellular calcium stores. The resulting increase in intracellular Ca^{2+} concentration activates calcium-release activated calcium (CRAC) channels in the cell membrane, resulting in an influx of more Ca^{2+} and the activation of calcineurin. Calcineurin dephosphorylates NFAT transcription factor family members, allowing their translocation to the nucleus (reviewed in Macian, 2005). DAG meanwhile activates the theta isoform of protein kinase C (PKCθ) in the immunological synapse, leading to phosphorylation of the inhibitor of NF-κB (IκB) protein, which normally binds Rel/NF-κB transcription factors and retains them in the cytoplasm. Upon phosphorylation, IκB is released and degraded, allowing translocation of the Rel/NF-κB transcription factors to the nucleus (reviewed in Ruland and Mak, 2003). CD28 also plays an important role in fully activating the Rel/NF-κB pathway, as without CD28 costimulation PKCθ does not relocate to the IS and become fully active (reviewed in Schmitz and Krappmann, 2006; Huang *et al*, 2002).

The signalling cascade initiated by TCR and CD28 engagement illustrates how an extracellular signal can lead to the translocation of transcription factors to the nucleus, where they may activate genes. B cell activation proceeds similarly, although the receptor molecules and associated tyrosine kinases are different. Briefly, antigen is bound by the B cell receptor (BCR) consisting of heavy and light chain immunoglobulins (Ig) on the surface of the B cell, causing clustering of the receptor complex and the formation of an IS (reviewed in Harwood and Batista, 2008). The IS also contains the Igα/Igβ proteins associated with the BCR, which upon clustering undergo phosphorylation of tyrosine residues in their cytoplasmic tails by Src-family kinases. These phosphorylated residues act as docking sites for more Src family members, which initiate a signalling cascade (reviewed in Gauld and Cambier, 2004). This cascade activates many of the same pathways seen in TCR signalling, such as Ca^{2+} and PKC, leading to the activation of the Rel/NF-κB (reviewed in Shinohara and Kurosaki, 2009), NFAT and activator protein 1 (AP-1) (de Gorter *et al*, 2007) transcription factors.

1.2 – Gene regulation

Control of gene transcription is important for the proper function of a cell, and therefore an intricate, multi-layered system of transcriptional control has developed in eukaryotes (reviewed in Venters and Pugh, 2009). As mentioned above, transcription factors can be activated by cell signalling pathways. Once in the nucleus, they bind to regulatory regions of DNA in a sequence specific manner and

affect gene expression. However, an additional level of control exists: epigenetic regulation. Here, the manner in which the DNA is packaged into the cell nucleus regulates access by transcription factors to their target DNA sequences, ensuring that only the correct genes will be activated in response to transcription factors (reviewed in Morse, 2007). While DNA sequence is identical between all cells of an organism, the epigenetic environment, or epigenome, is dynamic, changing both between cell types and within a single cell as it develops and responds to environmental stimuli, allowing diverse patterns of gene expression within a single organism (reviewed in Murrell *et al*, 2005).

1.2.1 - Gene regulation by transcription factor binding

As described above, activation of a signalling cascade results in the activation of a group of transcription factors in the cell nucleus. Once in the nucleus, these factors bind to the promoter and enhancer regions of genes in a DNA sequence-dependent manner, where they act to recruit co-activator or co-repressor proteins, thus determining the response of the gene to the signal cascade. In some cases these protein complexes form particular structures termed 'enhanceosomes', which are a specialised example of how transcription factors can co-operate to recruit co-activators and the transcriptional machinery (reviewed in Carey, 1998). One well characterised enhanceosome is the complex that forms at the interferon-β (*IFNβ*) enhancer prior to activation. Upon virus induction, *IFNβ* expression is upregulated. This requires the cooperative binding of a number of transcription factors to the enhancer region of the gene. The complex that forms is composed of a heterodimer

of the Rel/NF-κB family members, p50 and p65, an activating transcription factor 2 (ATF-2)/c-Jun heterodimer and interferon regulatory factor 1 (IRF-1). Protein-DNA and protein-protein interactions are stabilized by binding of high mobility group (HMG) I(Y) protein (Thanos and Maniatis 1995a; Thanos and Maniatis 1995b). The surface of the enhanceosome then serves to recruit a CREB binding protein/RNA polymerase II (CBP-PolII) complex, resulting in the formation of a preinitiation complex and gene transcription (Yie *et al*, 1999).

1.2.2 - Epigenetic mechanisms of gene regulation

However, transcription factors must interact with their cognate binding sites within the nuclear environment in which the DNA is highly compacted. Alterations in the manner in which DNA is packaged in the nucleus or covalent modification of the DNA bases themselves may affect the ability of transcription factors to access and bind to their DNA recognition sequences. These are generally referred to as epigenetic mechanisms, which can be defined as mitotically or meiotically heritable factors that affect gene expression without a change in the underlying DNA sequence (reviewed in Dupont *et al*, 2009), although the precise definition of epigenetics is still a matter of debate. Bird (2007) defines epigenetic events as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states". Regardless, the major mechanisms recognised as being involved in epigenetic regulation of gene expression are histone modifications and variations, DNA methylation, and chromatin remodelling. As will be outlined below, these

mechanisms interact with each other and transcription factors to create a delicately nuanced system of gene regulation.

Chromatin is the name given to the DNA-protein complex which serves to package DNA in the cell nucleus. The basic unit of chromatin is the nucleosome. This consists of a short (~145bp) region of DNA wrapped twice around an octamer of histone proteins: 2 each of histones H3, H4, H2A and H2B, arranged as an $(H3)_2(H4)_2$ tetramer bound to two H2A/H2B dimers (Luger *et al*, 1997). These histones in turn consist of a globular domain, which forms the core of the nucleosome, and an N-terminal "tail" that protrudes from the core. Nucleosomes are arranged along a strand of DNA with short stretches of linker DNA between them. This arrangement is commonly analogised as the "beads on a string" model. The linker histone H1 binds to the outside of the nucleosome and stabilises interactions between nucleosomes. Interactions between histone tails also contribute to the higher order structure of chromatin (Kornberg and Lorch, 1999; Arya *et al*, 2010). *In vitro*, nucleosomes condense into a 30nm diameter fibre secondary structure, although this is not observed in higher eukaryotes *in vivo*, and the exact organisation of the secondary and higher order structures *in vivo* is still a matter of debate (reviewed in Tremethick, 2007). However, it is clear that in the nucleus chromatin is a dynamic, heterogeneous structure, with areas of differing degrees of condensation. Two main forms of chromatin are recognised: euchromatin and heterochromatin. Euchromatin is considered to be less condensed, transcriptionally active chromatin. Heterochromatin, on the other hand, is highly condensed, tends to be gene poor and

those genes found within regions of heterochromatin also tend to be transcriptionally silent. Heterochromatin can be further divided into constitutive heterochromatin, which is condensed in all cell types (e.g. centromeres and telomeres), and facultative heterochromatin, which forms when genes are silenced during development and hence is cell-type specific (reviewed in Wegel and Shaw, 2005). However, these terms remain a simplification, as chromatin structure is dynamic, and may change in response to signals received by the cell.

1.2.3 - Histone modifications

One important factor in determining chromatin structure, and hence a key mechanism for epigenetic gene regulation, is covalent modification of histone tails. The N-terminal tails of the histone proteins contain many amino acid residues that can be targeted for covalent addition of various functional groups, such as methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation (adenosine diphosphate ribose) and SUMOylation (small ubiquitin-like modifier). Together, these marks are proposed to form a "histone code", in which the modifications on histone tails can be recognised and bound by other proteins (Strahl and Allis, 2000). In this model, establishment of histone marks can determine the recruitment of other activating or repressing factors to the chromatin and can therefore dictate gene activity. Two modifications in particular, acetylation and methylation, have been extensively studied, and their roles in gene regulation are now relatively well characterised.

Histone acetylation is generally, although not always, a mark of active or transcriptionally permissive chromatin (reviewed in Choi and Howe, 2009). Initially, the effect of acetylation on gene transcription was thought to be mainly due to non-specific disruptions of the higher order chromatin structure (van Holde and Zlatanova, 1996; Mizzen and Allis, 1998). Acetylation of positively charged lysine residues on the histone tails lessens their charge, weakening the interactions between the residues and the negatively charged DNA on adjacent nucleosomes and leading to a looser chromatin structure. In support of this, indiscriminate acetylation of a polynucleosome array, to the order of approximately 12 acetyl groups per histone octamer, was shown to inhibit higher-order structure formation and increase transcription from the template (Tse *et al*, 1998). Acetylation of lysine 16 on histone H4 (H4K16Ac) has also been shown to inhibit the formation of the higher-order 30nm chromatin fibre *in vitro* (Shogren-Knaak *et al*, 2006). However, as opposed to the indiscriminate effect observed by Tse *et al* (1998), this appeared to be a specific effect. Tremethick (2007) speculates that acetylation of H4K16 interferes with the interaction of the H4 tail with an acidic patch on adjacent nucleosomes, formed by seven amino acid residues on histone H2A. In this scenario, the specific acetylation of lysine residues is also important in determining chromatin structure. As opposed to these direct effects on chromatin structure, specific acetylation marks also have the potential to be recognised by and recruit various proteins that modify chromatin structure and function (reviewed in Kimura *et al*, 2005). The protein motifs that recognise acetylated lysine residues are known as bromodomains, and are found in a wide array of chromatin modifying proteins, including chromatin remodellers,

histone acetyltransferases, and a histone methyltransferase (reviewed in de la Cruz *et al*, 2005).

The processes of histone acetylation and deacetylation are controlled by two classes of proteins: the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). Not surprisingly, many HATs have been identified as transcriptional coactivators, such as p300/CBP and GCN5 (general control nonderepressible 5) (reviewed in Selvi and Kundu, 2009). HDACs, conversely, act as transcriptional corepressors (reviewed in Hildmann *et al*, 2007).

While increased histone acetylation is generally linked with gene activation, histone methylation can be associated with activation *or* repression, based on the specific residue that is methylated. Additionally, histone methylation may take place on arginine or lysine residues, and the same residue may be mono-, di- or, in the case of lysines, trimethylated (Zhang and Reinberg, 2001). Many studies have examined the distribution of histone methylation marks throughout the genome and their relevance to gene expression. High resolution analysis of histone methylation marks in human $CD4^+$ T cells in a genome-wide study revealed that monomethylated H3K27, H3K9 H4K20, H3K79 and H2BK5 were generally associated with the promoters of highly transcribed genes, while trimethylated H3K27, H3K9 and H3K79 were generally associated with repressed genes (Barski *et al*, 2007).

Since histone methylation appears to influence gene regulation in a highly specific manner, the associated histone methyltransferases (HMTs) are also highly specific. For example, H3K27 methylation is effected by the HMT enhancer of Zeste homolog 2 (EZH2), a member of the Polycomb Repressive Complex 2 (PRC2) (Cao *et al*, 2002), while suppressor of variegation 3-9 homolog 1 (SUV39H1) is responsible for H3K9 methylation (Rea *et al*, 2000). These marks can be recognised and bound by proteins containing a chromodomain motif (reviewed in Brehm *et al*, 2004). Recently, PRC2 was shown to bind to its own methylation marks, providing a mechanism for stable propagation and expansion of histone H3K27 di/trimethylation (Hansen and Helin, 2009). Histone demethylases also exist, such as jumonji domain-containing 3 (JMJD3), a specific demethylase of H3K27me3 (Xiang *et al*, 2007).

1.2.4 - Chromatin remodelling and nucleosomal dynamics

In addition to direct covalent modification of histones, the structure of chromatin can also be modified by chromatin remodellers: protein complexes that utilize ATP to physically move nucleosomes. The remodelling of nucleosomes is important in gene activation, as it allows access of coactivators and transcriptional machinery to the regulatory regions of the DNA.

Chromatin remodelling complexes (CRCs) are grouped into three major classes based on the ATPase subunit they contain. These classes are SWI2/SNF2 (switch/sucrose non-fermenting), ISWI (imitation switch), and Mi-2/CHD (chromodomain helicase DNA-binding) (reviewed in Vignali *et al*, 2000). The

SWI2/SNF2 CRCs were first identified in yeast, where two classes of complex, SWI/SNF and RSC (remodels structure of chromatin), are found, utilizing the Swi2/Snf2 and Snf2 homolog 1 (Sth1) ATPases respectively. In *Drosophila*, the analogous CRC is the Brahma complex, containing the eponymous Brm subunit. This nomenclature is extended to the mammalian SWI/SNF complexes, collectively known as BAF complexes, for Brg1/Brm associated factors. Brg1 (Brahma related gene) and Brm are two similar ATPase subunits, sharing approximately 70% homology. The SWI2/SNF2 ATPases are highly conserved across species, all containing a C-terminal bromodomain and other conserved regions of unknown function. Chromatin remodelling by SWI/SNF type CRCs has been shown *in vitro* to take place both in *cis*, by sliding nucleosomes along the DNA strand (Zhang *et al*, 2006), or in *trans*, where histones are evicted entirely from the DNA (Chandy *et al*, 2006; Gutiérrez *et al*, 2007).

The ISWI family of CRCs share an ATPase subunit that exhibits homology to the SWI2/SNF2 ATPases. However, this homology is only seen across the ATPase domain itself. In addition, the CRCs of this group are smaller, with less subunits than those in the SWI2/SNF2 group (reviewed in Längst and Becker, 2001).

The Mi-2 group CRCs are distinct in that these complexes exhibit histone deacetylase activity in addition to chromatin remodelling. The ATPase subunit Mi-2β shows some homology to Swi2/Snf2. Interestingly, Mi-2 CRCs have been found to contain proteins that bind to methylated DNA (Wade *et al*, 1999). Coupled with their ability

to deacetylate histones, this suggests that these complexes may play an inhibitory role in gene regulation, as methylated DNA is usually a mark of inactive genes (discussed in Chapter 1.2.5).

Unlike transcription factors, chromatin remodelling proteins generally lack DNA sequence specificity. Thus, the mechanism of CRC recruitment to target genes is complex, and generally involves protein/protein or DNA/protein interactions with subunits of the complex as well as the core ATPase component. For example, BAF complexes are large, usually containing 10-12 subunits besides Brg1/Brm that may modify interactions with DNA or other proteins. Subunits of the BAF complex such as BAF57, BAF250 and BAF60a are known to interact with nuclear hormone receptors (reviewed in Trotter and Archer, 2008). Additionally, BAF250 has been shown to specifically bind a pyrimidine-rich control element of the *δ-globin* gene (Nie *et al*, 2000), while BAF57 binds to 4-way junction DNA, a DNA structure similar to that seen in nucleosomes (Wang *et al*, 1998). BAF60a has also been shown to interact with the p53 transcription factor/tumour suppressor protein (Oh *et al*, 2008). Further, Brg1 itself may also play a role in BAF complex recruitment. The acetyl-lysine binding properties of the Brg1 bromodomain have been examined *in vitro* (Shen *et al*, 2007, Singh *et al,* 2007), where it was shown to bind acetylated lysine 9 and 14 of histone H3 (H3K9/14Ac2). Additionally, Brg1 has been reported to interact with a number of transcription factors, including Sp1 (Ma *et al*, 2004) and heat shock factor 4b (Hsf4b) (Tu *et al*, 2006).

Nucleosomal dynamics during gene transcription can be divided into three general events: remodelling of the promoter to allow initiation of transcription, disassembly and reassembly of coding region nucleosomes during elongation, and reassembly of histones at the promoter (reviewed in Workman, 2006). Removal of histones at a gene promoter is most likely governed by cooperation between CRCs and histone chaperones (Williams and Tyler, 2007). Histone chaperones are proteins that bind to histone proteins and mediate interactions between the histones and the DNA, regulating formation of the nucleosome (reviewed in Ransom *et al*, 2010). However, they do not remain bound to the nucleosome following assembly. The cooperation of CRCs and histone chaperones has been shown in several studies. Upon induction of the yeast homothallic switching endonuclease (*HO*) gene, the promoter is cleared of nucleosomes between -600 and -1800bp from the transcriptional start site. The Snf2 chromatin remodelling protein and antisilencing function 1 (Asf1) histone chaperone have been implicated in these nucleosome remodelling events. When Snf2 was rendered non-functional by point mutation, no chromatin remodelling was observed. Additionally, deletion of the histone chaperone Asf1 reduced chromatin remodelling and *HO* gene expression (Gkikopolous, 2009). Furthermore, during activation of the acid phosphatase (*PHO5*) gene, Asf1 was required for correct recruitment of SWI/SNF to the gene promoter, depletion of nucleosomes from the promoter and subsequent gene activation (Adkins *et al*, 2004; Adkins *et al*, 2007).

In contrast, the reassembly of histones during transcriptional downregulation has not been well studied, and the current literature on the subject is discussed in detail in

Chapter 5. During transcriptional elongation, histones are displaced from the gene ahead of the transcriptional machinery and rapidly reassembled behind the advancing PolII. At low levels of transcription, only the H2A/H2B dimers appear to be displaced and recovered. At high levels of transcription, though, the greater density of PolII on the gene can displace entire nucleosomes. The exact mechanisms are still under investigation, but appear to involve the FACT (facilitates chromatin transcription) histone chaperone, TFIIS elongation factor, and chromatin remodelling complexes (reviewed in Kulaeva *et al*, 2007).

1.2.5 - DNA methylation

One aspect of epigenetic regulation, DNA methylation, does not directly involve chromatin, and instead occurs through covalent modification of the DNA itself, i.e. methylation of specific cytosine residues. While several types of DNA methylation are known to exist, for example, CpT/A methylation in insects (Field *et al*, 2004) or CpNpG methylation in plants (Henderson and Jacobsen, 2007), the most common and well-understood form is CpG methylation. This involves the addition of a methyl group to the C5 carbon of a cytosine that is 5' of a guanine, known as a CpG dinucleotide, by a class of enzymes known as DNA-methyltransferases (DNMTs). This is always symmetrical, as the cytosines on both the sense and antisense strands of the DNA are methylated. When DNA replicates, the CpG dinucleotide on the newly synthesised strand is unmethylated. These hemimethylated strands are recognised and the CpG dinucleotide methylated by the maintenance methylase Dnmt1 (reviewed in Bestor, 2000). As well as maintenance methylation, *de novo*

methylation is also known to occur, in which a previously unmethylated CpG dinucleotide is targeted for methylation. Such *de novo* methylation is catalysed by the DNMTs Dnmt3a and Dnmt3b (Okano *et al*, 1999).

In the mammalian genome, some genes contain what are referred to as CpG islands: regions of several hundred to several thousand base pairs with enriched GC content and a large number of CpG sites (reviewed in Illingsworth and Bird, 2009; Bird, 2002). These islands are commonly found at the 5' end of genes, and are associated with 60-70% of human genes. A study by Zhu *et al* (2008) revealed that 79% of human housekeeping genes have a CpG island in their promoter, while the majority of tissue-specific genes do not. The CpG islands associated with housekeeping genes are generally hypomethylated. Some CpG islands, however, become hypermethylated during development and this is associated with gene silencing. Gene silencing by DNA methylation of CpG islands appears to be a method of stabilising the repression of genes which were previously silenced by other epigenetic mechanisms, and is thus often aberrantly regulated in cancer (Clark and Melki, 2002; Ohlsson and Kanduri, 2002).

DNA methylation may induce gene silencing through two different mechanisms: by recruiting repressive factors that work to maintain a transcriptionally silent environment, or by hindering the binding of transcription factors to the methylated DNA. Methylated DNA is recognised and bound by the methyl binding domain (MBD) proteins, which share the eponymous domain (reviewed in Fatemi and Wade,

2006). MBD proteins interact with many co-repressor proteins to silence gene expression (reviewed in Bogdanović and Veenstra, 2009). One well-characterised MBD family member recruited to methylated DNA is methyl CpG binding protein 2 (MECP2). MECP2 is known to specifically bind to CpG methylated DNA (Meehan *et al*, 1992) and has been shown to recruit the transcriptional corepressor mSin3A and the histone deacetylases HDAC1 and HDAC2 (Jones *et al* 1998; Nan *et al*, 1998), as well as associating with histone H3 lysine 9 methyltransferase activity (Fuks *et al*, 2003). However, recent work (Chahrour *et al*, 2008) indicates that MECP2 in brain tissue is also bound to transcriptionally active promoters, and associates with the transcriptional activator cAMP response element binding protein 1 (CREB1). Thus the role of MECP2 may go beyond that of just a simple repressor. As well as recruiting MBD proteins and their associated co-repressors, methylated DNA can also affect the binding of transcription factors to their recognition sites. A classical example of this is the CCCTC-binding factor (CTCF) transcription factor, which is unable to bind to its TF binding site when the DNA sequence is methylated. In the case of the insulin-like growth factor 2 (*Igf2*) and *H19* genes, which share a common locus, a CTCF binding region located between the two genes shows distinct differences in DNA methylation between the paternal and maternal alleles, which leads to parental imprinting of gene expression. In the paternal allele, the CTCF binding region is fully methylated, and CTCF cannot bind. This allows expression of the *Igf2* gene only from the paternal allele but blocks expression of *H19*. In the maternal allele, the CTCF region is unmethylated, allowing CTCF binding, blocking

of *Igf2* expression and activation of *H19* on the maternal allele only (Bell and Felsenfeld, 2000).

1.3 – Epigenetics in cytokine gene regulation

Apart from marking a gene as constitutively active or silent, epigenetic factors can also determine whether or not a gene will be expressed in a particular cell type in response to activation of signalling pathways. The role of these factors has been examined for a number of cytokines, with one excellent example being the contrasting sets of cytokines expressed by Th1 and Th2 helper T cells. These distinct types of T cell develop from naïve $CD4^+$ T helper (Thn) cells in response to different sets of immune challenge: viral and bacterial infection for Th1 cells, and asthma, allergies and helminth infection for Th2 cells (Abbas *et al*, 1996; O'Garra, 1998). Following differentiation, these cells exhibit distinct patterns of cytokine expression; upon stimulation, Th1 cells express *IFNγ* and *IL-2*, among others, while Th2 cells are characterised by inducible expression of *IL-4*, *IL-5*, and *IL-13* (Mosmann and Coffman, 1989). These differential expression patterns in response to antigenic stimulation have been shown to be established and maintained by epigenetic mechanisms as discussed above, including chromatin remodelling, DNA methylation, and histone modification (reviewed in van Panhuys *et al*, 2008; Aune *et al*, 2009). DNase hypersensitivity analysis of populations of Th1 and Th2 cell lines demonstrated that in Th1 cells, chromatin was accessible at the *IFNγ* locus, but not the *IL-4* locus. Conversely, in Th2 cells the opposite pattern was seen, with chromatin accessible at the *IL-4* locus but not the *IFNγ* locus. Primary splenic Thn

cells did not display accessibility at either locus, but when differentiated into either Th1 or Th2 cells in culture, the characteristic chromatin accessibility patterns seen in the Th1 and Th2 cell lines became established at the *IFNγ* and *IL-4* loci (Agarwal and Rao, 1998). Furthermore, differentiation into Th2 cells was additionally accompanied by CpG demethylation of the 5' region of the *IL-4* locus, which in Thn cells was hypermethylated (Lee *et al*, 2002).

Differential patterns of histone modifications were also established between Th1 and Th2 cells. Low levels of histone H3 and H4 acetylation at cytokine loci were observed in Thn cells. Following differentiation of the cells into either Th1 or Th2 populations, increases in histone acetylation were observed at subtype-specific loci. In Th1 cells, increases in H3 and H4 acetylation were observed at the *IFNγ* promoter but not the *IL-4* promoter, while in Th2 cells the *IL-4* promoter was acetylated and the *IFNγ* promoter was not (Fields *et al*, 2002). Repressive histone methylation marks have also been examined. In Th2 cells, the *IFNγ* locus is marked by the repressive H3K27me3 mark, while the *IL-4* locus is not (Chang and Aune, 2007). The opposing patterns of epigenetic regulatory factors and chromatin accessibility established at the *IL-4* and *IFNγ* genes during differentiation create a situation where activation of either gene by transcription factor binding is restricted to the appropriate cell type. Agarwal *et al* (2000) showed that the transcription factor NFAT1 binds to the *IFNγ* promoter in activated Th1 cells, and the *IL-4* promoter and distal enhancer in activated Th2 cells. No binding of NFAT1 was detected at the *IL-4* and *IFNγ* regulatory regions in stimulated Th1 and Th2 cells, respectively.

This illustrates how the response of an inducible cytokine gene to its activating stimulus can be predicted by the structure and modifications of the chromatin at the regulatory regions of the gene. An open chromatin structure, maintained by activating marks, facilitates transcription factor binding and transcription of the gene. However, not all inducible genes maintain an open chromatin structure at their promoters prior to gene induction. In many inducible genes, the promoter or other regulatory regions are assembled into nucleosomes prior to cell stimulation, and undergo chromatin remodelling upon stimulation, followed by gene transcription. This has been shown for the yeast genes *PHO5* and alkaline phosphatase (*PHO8*) (Adkins *et al*, 2004) as well as the mammalian *IFNβ* and *IL-12b* genes. During gene activation, the SAGA (Spt-Ada-Gcn5 acetyltransferase) HAT complex is recruited to the *PHO5* and *PHO8* promoters, resulting in hyperacetylation of promoter nucleosomes followed by chromatin remodelling. In *PHO8*, this remodelling is dependent on the SWI/SNF complex, while in *PHO5* deletion of the SWI/SNF Snf2 core subunit delays but does not eliminate *PHO5* promoter remodelling (Reinke *et al*, 2001; Reinke *et al*, 2003).

In the case of *IFNβ*, the enhancer is nucleosome free, but a nucleosome is positioned over the transcription start site (TSS). Upon stimulation, an enhanceosome of transcription factors forms on the nucleosome free enhancer region of the gene, as discussed earlier (Chapter 1.2.1). This enhanceosome then recruits the CBP and GCN5 HATs, which acetylate the nucleosome positioned over the TSS. The Brg1

chromatin remodelling protein is then recruited to this acetylated nucleosome via its bromodomain, allowing the remodelling of the nucleosome obscuring the TSS, and transcription of the gene (Agalioti *et al*, 2000, Agalioti *et al*, 2002).

IL-12 is a heterodimeric cytokine, composed of the p40 and p35 subunits, each encoded by an inducible gene. The gene encoding the p40 subunit, also known as *IL-12b*, undergoes remodelling of a single nucleosome covering the promoter in macrophages, following stimulation with lipopolysaccharide (Weinmann *et al*, 1999). Histone modifications have been implicated in the regulation of *IL-12b*. Severe sepsis was induced in a mouse model, leading to the depletion and subsequent recovery of dendritic cell populations. Following sepsis, *IL-12b* was downregulated in splenic dendritic cells. Chromatin immunoprecipitation (ChIP) analysis of histone methylation marks at the *IL-12b* promoter found high levels of H3K4me3, an active chromatin mark, and low levels of the repressive H3K27me2 mark in control animals. In animals that had undergone severe sepsis, the pattern was reversed, suggesting that these histone marks play a role in regulating inducibility of *IL-12b* (Wen *et al*, 2007).

Thus, epigenetic factors contribute to the formation of "permissive" and "repressive" chromatin states at inducible gene promoters. Permissive states allow gene transcription and, if necessary, chromatin remodelling of the promoter in response to activating stimuli, while a repressive state renders the gene insensitive to stimulation.

These states, when properly established, ensure correct patterns of inducible gene expression in response to activating stimuli.

1.4 – Granulocyte-macrophage colony stimulating factor

Granulocyte-macrophage colony stimulating factor, or GM-CSF, is a small glycoprotein approximately 22kDa in size, responsible for stimulating the production of several types of myeloid cell from $CD34^+$ progenitor cells, such as neutrophils, eosinophils, basophils (granulocytes) and monocytes, which mature into macrophages. As well as stimulating cell differentiation, it also inhibits the differentiation of $CD34^+$ cells into lymphoid progenitors or type 2 dendritic cells (reviewed in Martinez-Moczygemba and Huston, 2003). The effects, both stimulatory and inhibitory, of GM-CSF on immune cell production mean that GM-CSF is a crucial orchestrator of the immune response. Expression of *GM-CSF* is relatively widespread, and can be induced in T cells, mast cells, macrophages, fibroblasts and endothelial cells, among others (Gasson *et al*, 1991).

GM-CSF has been implicated as a potential causative or exacerbating factor in several disease states. Elevated levels of GM-CSF protein were detected in the sputum of asthma and chronic obstructive pulmonary disease (COPD) sufferers, with the level of GM-CSF positively correlating with disease severity. Additionally, GM-CSF and GM-CSF receptor protein levels were elevated in the bronchial submucosa cells of severe asthma sufferers, but not COPD sufferers (Saha *et al*, 2009). Constitutive expression of *GM-CSF* has also been reported in acute myeloblastic

leukaemia (Young *et al*, 1987). Furthermore, GM-CSF, in combination with the closely related granulocyte colony stimulating factor (G-CSF), promoted growth and malignancy of cell lines derived from human squamous cell carcinomas (Gutschalk *et al*, 2006).

The *GM-CSF* gene is located on chromosome 5 in humans, and chromosome 11 in mice, sharing a locus with *IL-3*. The *GM-CSF* promoter is a region of approximately 100bp directly upstream of the TSS. Additionally, an enhancer region is found 3kb (human) or 2kb (murine) upstream of the TSS (reviewed in Cockerill, 2004). The enhancer plays an important role in the regulation of the human *GM-CSF* gene (Cockerill *et al*, 1999); however, in mice, there is evidence that the promoter is the dominant regulatory region (Osborne *et al*, 1995). The *GM-CSF* promoter contains two distinct transcription factor binding regions, termed the CD28 response region (CD28RR) and the Conserved Lymphokine Element 0 (CLE0), each of which responds to a different activating signal. These two regions are themselves composed of binding sites for several transcription factor complexes. The CD28RR contains three distinct transcription factor binding motifs: a 10bp element known as the CD28 response element (CD28RE) or cytokine-1 (CK-1), which binds variant Rel/NF-κB family member dimers, an adjacent classic NF-κB binding site, and an Sp1 binding site (Shang *et al*, 1999). The CLE0 consists of binding sites for the transcription factors NFAT, AP-1, and E-twenty six (Ets) (Jenkins *et al*, 1995). A schematic of these binding sites is shown in Figure 1.1. The pathways that activate the NFAT and Rel/NF-κB transcription factors can be activated by engagement of the TCR and

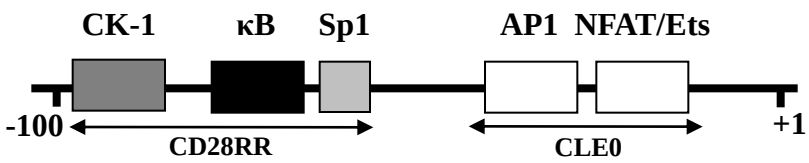

Figure 1.1: The *GM-CSF* proximal promoter. The region depicted is directly 5' of the transcriptional start site (+1). CD28RR, CLE0 and binding sites for transcription factors are indicated schematically.

costimulation by CD28, or engagement of the BCR by antigen, as discussed above (Chapter 1.1.1).

1.4.1 - NFAT

The NFAT (nuclear factor of activated T cells) family comprises five members – NFAT1 (NFATp), NFAT2 (NFATc), NFAT3, NFAT4 (NFATx) and NFAT5. Although first discovered in T cells, as the name suggests, they have a widespread expression pattern. It is in the immune system, however, that their action is best understood. All members of the family share a transactivating NFAT homology region (NHR), and a DNA-binding domain that bears homology to that of the Rel/NF-κB proteins (Rel homology domain or RHD). Additionally, all but NFAT5 are regulated by calcium signalling. Briefly, activation of receptors on the cell surface involved in the calcium signalling pathway leads to production of IP_3. IP_3 then causes intracellular stores of calcium to be released, which in turn activates calcium channels on the cell membrane. The increased intracellular levels of calcium lead to the activation of the phosphatase calcineurin, which dephosphorylates serine residues in the NHR and causes translocation of NFAT to the nucleus (Macian, 2005).

NFAT-dependent activation is seen for many cytokine genes, including *IL-2* (Shannon *et al*, 1995), *IFN-γ* (Kiani *et al*, 2001) *IL-4* and *TNF-α* (Luo *et al*, 1996) as well as *GM-CSF*. Both NFATc and NFATp are known to be important in activating transcription of the *GM-CSF* gene (Shang *et al*, 1999). Cockerill *et al* (1993) showed

that treatment with Cyclosporin A (CsA), an inhibitor of NFAT, blocks *GM-CSF* gene activation. Furthermore, NFAT/AP-1 complexes have been shown to bind to the *GM-CSF* promoter element (Tokumitsu *et al*, 1993, Tsuboi *et al*, 1994).

1.4.2 - Sp1

The specificity protein (Sp) family of transcription factors contains four members: Sp1, Sp2, Sp3 and Sp4. These bind GC-rich regions in gene promoters by means of a conserved three zinc finger domain. While little is known about the roles of Sp2 and Sp4, Sp1 and Sp3 are ubiquitously expressed in eukaryotic cells, and known to be involved in the regulation of many genes. The consensus Sp1 binding site is 5' GGGGCGGGG 3' (reviewed in Li *et al* 2004), and Sp1 has been shown to bind to this recognition site even when DNA is assembled into nucleosomes (Li *et al*, 1994).

Studies have shown that Sp1 is important in the regulation of *GM-CSF* gene expression. Depletion of Sp1 from nuclear extracts blocked transcription from a *GM-CSF* template *in vitro* (Masuda *et al*, 1994). Kochetkova *et al* (1997) showed that formation of a DNA triple helix over the CD28RR by introduction of an oligonucleotide blocked Sp1 binding to a probe containing the *GM-CSF* promoter sequence in gel shift assays and inhibited *GM-CSF* gene transcription *in vivo*. However, the oligonucleotide used to form the triple helix also covered the NF-κB site that is adjacent to the Sp1 site, and blocked NF-κB binding to the promoter in gel shift assays, making it difficult to determine the contribution of each site to the observed effect on transcription.

1.4.3 - Rel/NF-κB

The Rel/NF-κB (nuclear factor kappa B) family of transcription factors is a group of proteins related by the conserved Rel homology domain (RHD), which is involved in DNA binding and dimerisation. Family members can be broadly divided into two classes based on the identity of the region C-terminal of the RHD. One class contains multiple ankyrin repeats, which are cleaved to activate the protein. This class includes p105/p50 and p100/p52 (where the second name indicates the cleaved, active form). The second class contain a C-terminal transactivation domain, and includes c-Rel, RelA (p65), and RelB (reviewed in Gilmore, 1999). The members of this family are known to play a very important role in the activation of immune gene expression. Rel/NF-κB family members act to stimulate gene expression by binding to 10bp regions of DNA as either homo- or heterodimers. The "classic" dimer, generally known just as NF-κB, is a heterodimer of the family members p50 and RelA. However, other dimers also exist (reviewed in Sun and Anderson, 2002).

Regulation of Rel/NF-κBs is achieved by association with the inhibitory IκB proteins. These proteins bind to Rel/NF-κB dimers in the cytoplasm, obscuring the nuclear localisation sequence (NLS) of the Rel/NF-κB proteins and preventing nuclear translocation. When a stimulatory signal is received, IκB is phosphorylated by the kinase IKK, targeting it for degradation. This exposes the NLS and allows translocation of the Rel/NF-κB dimer to the nucleus, where it binds the target DNA region (reviewed in Caamaño and Hunter, 2002).

Rel/NF-κB family members are known to be important in the activation of the *GM-CSF* gene. As shown in Figure 1.1, the *GM-CSF* promoter CD28RR contains both a classic NF-κB binding site (κB), and the CK-1 or CD28 response element (CD28RE) site. Schreck *et al* (1990) showed that Rel/NF-κB proteins bind to oligonucleotide probes containing these sequences. Using gel shift assays, it was discovered that the classic site binds a p50/RelA dimer, while the CK-1 binds c-Rel containing complexes, and RelA homodimers (Himes *et al*, 1996). Additionally, complexes containing p52 and c-Rel extracted from stimulated T cells were shown to bind to the promoter CK-1 sequence (Kahn-Perlès *et al*, 1997). As mentioned above (Kochetkova *et al*, 1997), formation of a DNA triplex on the *GM-CSF* gene promoter by binding a DNA oligonucleotide to the CD28RR blocks Rel/NF-κB binding to the promoter and *GM-CSF* gene transcription. The CK-1 sequence is found in a number of cytokine promoters in a highly conserved fashion, and studies have shown that in the cases of *G-CSF* and *IL-2*, RelA binds this region and contributes to activation of these genes (Dunn *et al*, 1994; Lai *et al*, 1995).

1.4.4 - Epigenetic regulation of the *GM-CSF* promoter

Chromatin remodelling is known to play a key role in activating expression of the *GM-CSF* gene, as a variety of studies over the years have shown. Initially, this was noticed at the enhancer region of the human gene, where an inducible DNase I hypersensitive site was observed at the 3kb upstream enhancer following T cell stimulation (Cockerill *et al*, 1993). Treatment with CsA prevented the appearance of

this site, implicating NFAT as a factor in enhancer remodelling. This inducible site was further shown to only appear in human cells capable of expressing the *GM-CSF* gene (Cockerill *et al*, 1999). A DNase I hypersensitive site was also noted to form at the promoter following stimulation in some, but not all, of the *GM-CSF* expressing cell lines studied. It was subsequently shown (Holloway *et al*, 2003) that chromatin remodelling of the murine promoter in activated EL-4 T cells is confined to the region -174 to +24 (where +1 is the TSS), consistent with remodelling of a single nucleosome covering the proximal promoter.

These chromatin remodelling events were subsequently found to be distinct from transcriptional activation of the gene, displaying differing signalling and transcription factor requirements. Remodelling of the promoter is dependent on Rel/NF-κB proteins. Cakouros *et al*, (2001) demonstrated that mutation of the κB site in the *GM-CSF* promoter to a second CK-1 site prevented appearance of a DNase I hypersensitive site at the promoter upon stimulation, while Holloway *et al* (2003) showed that promoter chromatin remodelling upon stimulation was reduced in EL-4 T cells expressing a mutant IκB, which cannot be phosphorylated (thereby preventing the translocation of Rel/NF-κB proteins to the nucleus). Gene transcription, on the other hand, was demonstrated to require both the Rel/NF-κB and NFAT family proteins (Brettingham-Moore *et al*, 2005). The chromatin remodelling events that occur at the promoter have been found to involve the depletion of histones from the promoter (Chen *et al*, 2005). It is likely that the Brg1 chromatin remodelling protein plays a role in these events as Brg1 is associated with the *GM-CSF* promoter in non-

stimulated T cells. Upon T cell stimulation, both Brg1 and histone proteins are lost from the promoter region of the gene concurrently with an increase in accessibility, indicating loss of the nucleosome. Expression of a Brg1 mutant lacking ATPase activity was shown to reduce both promoter remodelling and *GM-CSF* transcription. However, in the A20 B cell line, which does not express *GM-CSF* in response to stimulation, Brg1 was not enriched at the *GM-CSF* promoter, and chromatin remodelling was not observed upon stimulation (Brettingham-Moore *et al*, 2008). This suggests that *GM-CSF* promoter chromatin remodelling upon stimulation is mediated by the BAF complex.

These observations have enabled a model for *GM-CSF* gene activation in T cells to be generated (Figure 1.2). In non-stimulated cells, the *GM-CSF* promoter is covered by a nucleosome, and marked by enriched Brg1 binding. Upon activation of the TCR and CD28 signalling pathways, Rel/NF-κB proteins translocate to the nucleus, resulting in chromatin remodelling events involving loss of Brg1 and histones from the *GM-CSF* promoter, allowing gene transcription to occur. However, in B cells, which do not express *GM-CSF* in response to stimulation, remodelling upon stimulation does not occur, and Brg1 is not enriched at the *GM-CSF* promoter. Based upon the available evidence and what is known from the literature, it is likely that epigenetic factors may be responsible for the difference in *GM-CSF* gene inducibility between T and B cells.

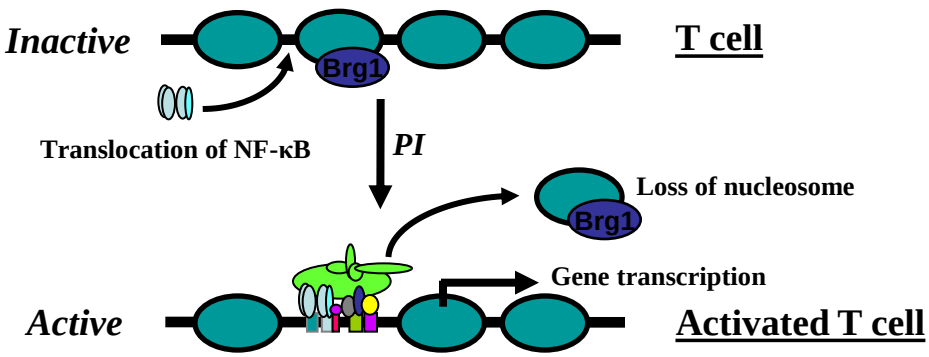

Figure 1.2: Model of *GM-CSF* gene activation in T cells. In unstimulated EL-4 T cells, the *GM-CSF* promoter is covered by a nucleosome. The Brg1 chromatin remodelling protein is associated with the promoter. Upon stimulation with PMA and ionophore (PI) and activation of the Rel/NF-κB proteins, the promoter nucleosome undergoes remodelling and is lost from the promoter along with Brg1, allowing gene transcription to occur.

1.5 - Hypothesis and aims

The hypothesis underlying this thesis is that differential patterns of epigenetic marks are established at the *GM-CSF* promoter between different cell types, and dictate the ability of the promoter nucleosome to undergo chromatin remodelling in response to a stimulus, resulting in gene activation. The aims of this thesis, therefore, were:

1. To characterise the epigenetic factors present at the *GM-CSF* promoter in T and B cells, and;

2. To investigate the role these factors play in predicting the response of the *GMCSF* gene to an activating stimulus.

Chapter Two - Materials and Methods

2.1 – Cell culture

2.1.1 – Culture of cell lines

Murine EL-4 T cells were cultured in Royal Menlo Park Institute (RPMI) 1640 medium (Gibco BRL) supplemented with 10% foetal calf serum (FCS) (Sigma), 100U/mL penicillin and 100µg/mL streptomycin (JRH Biosciences). Murine A20 and WEHI-231 B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) supplemented as above, with the addition of 0.05% β-mercaptoethanol. All cell lines were maintained in an environment of 5% CO_2 and 37ºC. Cell density was measured by counting trypan blue stained cells using a haemocytometer, and adjusted to $2x10^5$ cells/mL every 24 hours (EL-4 and A20) or 48-72 hours (WEHI-231) by addition of fresh medium.

2.1.2 - Thawing cell lines

A vial of $1x10^7$ cells from -80°C or liquid nitrogen storage was thawed in a 37°C water bath, and transferred to a centrifuge tube. Ten mL of medium was added and the cells pelleted at 500g for 5 minutes. The supernatant was removed, the cells resuspended in 1mL medium and added to a flask containing 9mL medium.

2.1.3 - Freezing cell lines

Cells at a concentration of $1x10^7$ cells/mL had an equal volume of medium containing 20% DMSO slowly added. One mL aliquots were transferred to cryovials, which were then wrapped in cotton wool to allow gradual freezing, and stored at -80°C or the vapour phase of liquid nitrogen.

2.1.4 - Transient cell transfection and luciferase assay

EL-4 T cells or A20 B cells ($4.5x10^6$) in 300µL media containing 20% FCS were transfected with 5µg-10µg plasmid at 270V and 975µF using a Bio-Rad Gene Pulser X Cell. At 24 hours post-transfection, cells were either PI stimulated or left unstimulated for 8 hours, and collected by centrifugation at 500g for 5 minutes. Total protein was isolated by lysis in 1x Luciferase Cell Culture Lysis Reagent (Promega), quantitated by Bio-Rad Protein Assay according to the manufacturer's instructions, and 30µg protein analysed for luciferase activity using a Promega Luciferase Assay Kit and Turner Biosystems Veritas™ Microplate Luminometer. Depending on the amount of protein recovered, up to three separate analyses of each sample were performed, and the results averaged.

2.1.5 – Generation of stably transfected cells

EL-4 T cells were transfected as in 2.1.4 with 10µg of linearised plasmid (pBSXH10.5GMWT, pBSXH10.5GMSp1m, or pBSXH10.5GMCD28RRm) and 1µg linearised pcDNA3.1 plasmid containing the neomycin resistance gene. At 24 hours post-transfection, cells were treated with 600ng/mL G418 (Sigma) alongside an

untransfected control. Cell density was monitored by trypan blue staining and haemocytometer counting. When all untransfected control cells had died, and cell growth became exponential in the transfected samples, G418 concentration was reduced to 400ng/mL to maintain the cultures.

2.1.6 - Primary T and B cell isolation

Ethics approval was sought and gained from the University of Tasmania Animal Ethics Committee (project number A0009765). Spleens were isolated from 8-week old male C57/Bl6 mice and cells dissociated by passing through a 70µm cell strainer. Mononuclear cells were collected using density gradient centrifugation through Lympholyte-M (Cedarlane) according to the manufacturer's instructions. Cells were then incubated with antibody-conjugated magnetic beads (Miltenyi Biotec) against either CD4 (T cells) or CD19 (B cells), and positive cells purified by magnetic separation, according to the manufacturer's instructions. Purified T or B cells were collected in DMEM supplemented with 10% FCS, 100U/mL penicillin, 100µg/mL streptomycin, 0.05% β-mercaptoethanol, 1mM sodium pyruvate and 10mM HEPES. During experimental treatment, cells were maintained at 37ºC in 5% CO_2.

2.1.7 - Cell treatments

When required, cell cultures were treated with various reagents as outlined in Table 2.1. Treatment times are specified in the relevant chapters.

Reagent	Stock concentration (solvent)	Final concentration in cell culture
Phorbol myristate acetate (PMA)	1mg/mL (DMSO)	20ng/mL
Calcium ionophore (I)	10mM (DMSO)	1µM
5-aza-2-deoxycytidine (aza)	5mg/mL (DMSO)	0.25µM
Trichostatin A (TSA)	1mg/mL (DMSO)	200ng/mL
Cycloheximide (CHX)	100mg/mL (ethanol)	10µg/mL

Table 2.1: Reagents used for cell treatment

2.2 – Gene expression analysis

2.2.1 - RNA extraction

Total RNA was isolated from cells as follows. Cells ($5x10^6$) were pelleted by centrifugation (500g, 5 minutes) and lysed in 1mL TRI-Reagent (Sigma). Chloroform (200µL) was added and the sample mixed. Following a ten minute incubation at room temperature, the sample was centrifuged for 15 minutes at 12,000g at 4ºC, and the colourless upper layer removed to a fresh microcentrifuge tube. Isopropanol (500µL) was added, the sample mixed and incubated at room temperature for 30 minutes to precipitate RNA. Precipitated RNA was pelleted by centrifugation (10 minutes, 13,000g, 4ºC) and the supernatant removed. The RNA

pellet was washed with 1mL 70% ethanol (5 minutes, 13,000g, 4ºC), air dried, and resuspended in 50µL MilliQ water.

2.2.2 - cDNA synthesis

Total RNA was quantitated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). RNA (1µg) was treated with 1 unit of DNase I (Sigma) at 37ºC for 30 minutes in 1x First Strand Buffer (Invitrogen), followed by 5 minutes at 75ºC to inactivate DNase. Oligo dT (0.1nmol, Sigma) was added and the sample incubated at 70ºC for 10 minutes. mRNA was then reverse transcribed to cDNA by treating with 100 units of Superscript Reverse Transcriptase (Invitrogen) in 1x first strand buffer (Invitrogen) supplemented with 0.1M DTT (Invitrogen) and 0.5mM dNTPs (Promega) at 42ºC for 50 minutes followed by 70ºC for 15 minutes. For qPCR analysis, cDNA was diluted 1:5 to 10ng/µL, and 50ng used per reaction.

2.2.3 - qPCR

qPCR was performed using the QIAGEN QuantiTect SYBR Green PCR kit. Each reaction contained 12.5µL QuantiTect SYBR Green, 4.5µL RNase-Free Water, and 1.5µL each of the appropriate forward and reverse primer (5µM stocks). In each reaction 5µL of DNA was added. In most cases this represented 50ng of DNA, except in ChIP-PCR where 5µL of resuspended DNA was added. Primers used are described in Table 2.2. Cycling was performed on a Corbett Rotor Gene with the following profile:

Activation: 95ºC for 15 minutes

Cycle: 94ºC for 15 seconds, 60ºC for 60 seconds, 40 cycles

Melt: Ramp from 65ºC to 95ºC, 1ºC/5 seconds

Primer		Sequence (5' → 3')	Target
mGM promoter or CpG -1	For	GCCTGACAACCTGGGGGAAG	Mouse *GM-CSF* promoter/ CpG dinucleotide -1
	Rev	TGATTAATGGTGACCACAGAACTC	
mGM-RNA	For	AAGGTCCTGAGGAGGATGTG	Mouse *GM-CSF* mRNA
	Rev	GAGGTTCAGGGCTTCTTTGA	
mGAPDH	For	AAGTATGATGACATCAAGAAGGTGGT	Mouse *GAPDH* mRNA
	Rev	AGCCCAGGATGCCCTTTAGT	
1.1kb 5'	For	GAGCTTCTGGAGAGGGAGGT	Region 1.1kb upstream of the *GM-CSF* gene
	Rev	TCCCAGGCTTAGTCTGTTGC	
8kb 5'	For	CTCATATGGAAGGCCCAAGT	Region 8kb upstream of the *GM-CSF* gene
	Rev	GGAGCTACAGGCAGTTGTGA	
RhoD	For	ATATCTCGCGGATGCTGAAT	Mouse *Rhodopsin* promoter
	Rev	GACAGAGACCAAGGCTGCTT	
CpG+1	For	AAGGTCCTGAGGAGGATGTG	Mouse CpG dinucleotide +1 (see Fig. 3.3a)
	Rev	GAGGTTCAGGGCTTCTTTGA	
CpG-2	For	AAAAGGAGAGGCTAGCCAGA	Mouse CpG dinucleotide -2 (see Fig. 3.3a)
	Rev	TAAGCCCTTCCAAGAACTGG	
CpG-3	For	GAACAGCAGGTGCTATGGAA	Mouse CpG dinucleotide -3 (see Fig. 3.3a)
	Rev	GGCATATTTGGATTTCCTGG	
CpGEn	For	GGAAACTCCTTCCAGAGGGTT	Mouse CpG dinucleotide in *GM-CSF* enhancer (see Fig. 3.3a)
	Rev	TGGGGCTGTGGCAGGGC	
mIL-2	For	CCTGAGCAGGATGGAGAATTACA	Mouse *IL-2* mRNA
	Rev	TCCAGAACATGCCGCAGAG	
hGM promoter	For	GCCTGACCACTAGGGAAAAG	Human *GM-CSF* transgene promoter
	Rev	TGATTAATGGTGACCACAGAACTC	
hGM-RNA	For	CACTGCTGCGAGATGAATGAAA	Human *GM-CSF* transgene mRNA
	Rev	GTCTGTATCCAGCTCGGCTC	

Table 2.2: Primers used for qPCR analysis

2.3 – Protein analysis

2.3.1 - Isolation of nuclear proteins

Nuclear extracts were prepared by a modification of the method of Schreiber *et al* (1989). Briefly, cells ($1.25x10^7$) were pelleted in a pre-chilled 50mL Falcon® tube

(500g, 5 minutes, 4°C), and the supernatant removed. The cell pellet was washed in 10 mL PBS per $1.25x10^7$ cells and the cells pelleted as above. The rest of the protocol was carried out in a cold room (4°C). The cell pellet was resuspended in 1mL of Buffer A per $1.25x10^7$ cells (10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA pH 8, 0.5% Igepal). The sample was transferred to a microcentrifuge tube (pre-chilled), incubated on ice for 5 minutes, and centrifuged at 3000g for 5 minutes. The pellet was resuspended in 1mL of Buffer A (without Igepal) per $1.25x10^7$ cells, and centrifuged at 3000g for 5 minutes. The supernatant was removed and the nuclear pellet resuspended in 25µL of Buffer C (400mM NaCl, 7.5mM $MgCl_2$, 0.2mM EDTA pH 8, 1mM DTT, supplemented with Complete EDTA-Free Protease Inhibitor Cocktail (Roche)). The resuspended pellet was incubated on ice for 15 minutes with mixing and then centrifuged at 13000g for 5 minutes. The supernatant containing nuclear proteins was stored at -80°C.

2.3.2 - SDS-PAGE

A 10% polyacrylamide gel was prepared with a 3% polyacrylamide stacking gel. Protein samples to be analysed were diluted in 4x Sample Buffer (40% glycerol, 240mM Tris•HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol). The samples were heated at 95°C for 5 minutes, loaded onto the gel along with 10µL Prestained Protein Ladder (Invitrogen) and electrophoresed in 1x SDS-PAGE Running Buffer (25mM Tris, 192mM glycine, 0.1% SDS) for 75 minutes at 125V.

2.3.3 - Western blot

The gel was removed from the SDS-PAGE apparatus, assembled into the western blotting apparatus, and protein transferred onto a nitrocellulose membrane in Western Transfer buffer (20mM Tris, 150mM glycine, 20% methanol, 0.1% SDS) for 18 hours at 20V. The nitrocellulose was removed from the transfer apparatus and placed in blocking solution (Blocking Reagent (Roche) diluted 1:5 in 1x TNT (10mM Tris, 150mM NaCl, 0.05% Tween 20) for one hour with shaking at room temperature. It was then incubated with primary antibody diluted (see Table 2.3 for antibody dilutions) in 1x TNT containing 0.5% blocking solution and again incubated as above. The blot was then washed for 3 x 10 minutes in 1x TNT, incubated with secondary antibody diluted in 1x TNT containing 0.5% blocking solution for another hour, and washed. The protein/antibody complex was detected using SuperSignal® West Pico Luminol enhancer solution and stable peroxide solution (Pierce), visualised on high performance autoradiography film. For analysis with additional antibodies, the blot was placed in stripping buffer (62.5mM Tris, 2% SDS, 0.7% β-mercaptoethanol) and incubated with shaking at 65°C for 30 minutes. It was then washed (3 x 10 minutes) in 1x TNT, and placed in blocking solution for one hour as above.

Antibody	Dilution factor	Supplier
α-c-Rel (primary)	1:1000	Santa Cruz
α-RelA (primary)	1:1000	Santa Cruz
α-Mi-2β (primary)	1:1000	Provided by Prof. Bob Kingston, Harvard Medical School
α-AcH3 (primary)	1:1000	Upstate Biotech
α-Sp1 (primary)	1:2000	Santa Cruz
α-rabbit HRP (secondary)	1:2000	Dako

Table 2.3: Antibodies and dilution factors for western blot analysis

2.4 - Chromatin analysis

2.4.1 - Chromatin immunoprecipitation (ChIP)

Cells ($5x10^6$) were treated with formaldehyde at a concentration of 1% to crosslink proteins and DNA for 15 minutes at room temperature with agitation. Glycine was added to a final concentration of 0.125M to quench the cross-linking reaction and a further ten minute incubation with agitation performed. Cells were then pelleted (500g, 5 minutes) and washed twice with ice-cold 1x PBS. $2x10^6$ cells in 1x PBS were resuspended in 250µL SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris) and incubated on ice for ten minutes.

Following lysis, the sample was sonicated 4 times (30 second pulse, setting 3) with a Microson XL 2000 sonicator to shear the DNA/protein complexes into fragments approximately 200-1000bp in length. The sample was chilled on ice between pulses. One mL of ChIP dilution buffer (0.01% SDS, 1.2mM EDTA, 16.7mM Tris•HCl, 1% Triton X-100, 167mM NaCl) and 60µL Protein A Agarose/Salmon Sperm DNA

slurry (Upstate) were added and the sample incubated for 2 hours at 4ºC on a rotating wheel. The slurry was precipitated for 1 minute at 2000g, and the supernatant aliquoted depending on the antibody used, as outlined in Table 2.4.

Antibody (supplier)	Vol. supernatant aliquoted	Amount antibody used
α-H3 (Upstate)	450µL	2µL (1.8µg)
α-AcH3 (Upstate)	200µL	4µL (4µg)
α-H3K27me3 (Upstate)	200µL	4µL (4µg)
α-Sp1 (Santa Cruz)	450µL	10µL (2µg)
α-Mi2 (Prof. Bob Kingston, Harvard Medical School)	450µL	5µL (serum antibody)
α-Brg1 (Santa Cruz)	450µL	5µL (1µg)

Table 2.4: Supernatant volumes and antibodies used for ChIP analysis

For each antibody (Ab) sample, a no-antibody (No Ab) control of the same volume was also aliquoted. Additionally, 100µL of supernatant as the total input (TI) control was stored at -80ºC. Ab and No Ab samples were diluted to 1mL by the addition of ChIP dilution buffer, the appropriate amount of antibody (indicated in Table 2.4) added to the Ab sample, and the samples incubated overnight at 4ºC on a rotating wheel.

The following day, 60µL of Protein A Agarose/Salmon Sperm DNA slurry was added to all samples, followed by a further 4 hour incubation at 4ºC on a rotating wheel. The slurry (bound to the DNA/protein/antibody complexes) was pelleted by centrifugation at 2000g for 1 min at 4ºC and the supernatant discarded. The slurry was then washed with 1mL each of low salt buffer (2mM EDTA pH 8, 0.1% SDS, 1% Triton X-100, 20mM Tris•HCl pH 8.1, 150mM NaCl), high salt buffer (as for low salt except 500mM NaCl), LiCl buffer (1mM EDTA pH 8, 10mM Tris•HCl pH 8.1, 250mM LiCl, 1% Igepal, 1% sodium deoxycholate) and TE (1mM EDTA pH 8,

10mM Tris•HCl pH 8.1). Following these washes, DNA/protein complexes were eluted from the slurry with 200µL elution buffer (100mM $NaHCO_3$, 1% SDS). The samples were incubated on a rotating wheel for 15 minutes at room temperature, the slurry pelleted and the supernatant containing eluted DNA/protein complex transferred to a clean microcentrifuge tube. This elution step was performed twice per sample and the eluates combined. Eluates, along with the TI samples, were treated with 1µL of 20mg/mL Proteinase K (Qiagen) and 0.1 vol 4M NaCl at 65ºC overnight to reverse protein/DNA crosslinks.

DNA was subjected to phenol/chloroform extraction and recovered by ethanol/sodium acetate precipitation. Precipitated DNA was washed with 1mL 70% ethanol, vortexed briefly, and centrifuged at 13,000g for 15 minutes. DNA pellets were then air-dried and resuspended in 50µL MilliQ water. For qPCR analysis, 5µL of each sample (Ab, No Ab and TI) was used.

2.4.2 - Chromatin accessibility by real time PCR (CHART-PCR)

The CHART-PCR assay was performed as described in Rao *et al*, 2001. Briefly, cells ($1x10^7$) were lysed in 1mL chilled nuclei buffer (10mM Tris pH 7.5, 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA pH 8, 0.5% Igepal, 0.15mM spermine, 0.5mM spermidine) and centrifuged at 3000g for 5 minutes. The nuclei were washed once with 1mL chilled micrococcal nuclease (MNase) buffer (10mM Tris pH 7.5, 15mM NaCl, 60mM KCl, 0.15mM spermine, 0.5mM spermidine), centrifuged at 3000g for 5 minutes and resuspended in 200µL MNase buffer. Nuclei (94µL) were incubated

with 25U MNase (Roche) and 1µL 0.1M $CaCl_2$ for 5 minutes at room temperature, with a non-MNase treated control incubated in parallel. The reaction was quenched with 20µL stop buffer (0.1M EDTA pH 8, 0.05M EGTA pH 8), and the total volume adjusted to 200µL by addition of MNase buffer. DNA was then extracted from each sample using the QIAGEN® Blood Kit according to the manufacturer's instructions. DNA was quantitated, diluted to 10ng/µL, and 50ng used in a qPCR reaction with primers against the region of interest.

2.4.3 - Methylation assay

Cells to be analysed (2.5-5x10^6 cells) were pelleted at 500g and resuspended in 200µL 1x PBS. DNA was extracted from each sample using the QIAGEN® Blood Kit according to the manufacturer's instructions. 1µg of DNA was treated with 10 units of *Aci*I (NEB) in 0.1mg/mL BSA (NEB) and 1x Buffer 3 (NEB) in a total volume of 20µL. In parallel, a "mock" reaction was set up substituting 1µL 50% glycerol in place of *Aci*I. The samples were incubated at 37ºC overnight. For qPCR analysis, 80µL MilliQ water was added to each sample to dilute the DNA to a concentration of 10ng/µL, and 50ng used in qPCR analysis with primers against the site of interest. Percent methylation at each site was calculated as ((copies AciI)/ (copies mock)) x 100.

2.5 – Microbiological and general molecular biology techniques

2.5.1 – Preparation of L-broth and L-agar plates

L-broth was prepared by dissolving 5g tryptone, 2.5g yeast extract and 2.5g NaCl per 500mL MilliQ water. 200µL 5M NaOH was added and the broth autoclaved. For L-agar plates, 1.5g agar per 100mL broth was added prior to autoclaving. Once the agar solution had cooled sufficiently, ampicillin was added (if necessary) to a concentration of 100µg/mL and the plates poured in sterile conditions.

2.5.2 - Transformation of competent cells

Plasmids to be amplified were transformed into One Shot Top10 Competent Cells (Invitrogen) according to the manufacturer's instructions. Transformed cells were plated onto L-agar plates containing 100µg/mL ampicillin and grown at 37ºC overnight.

2.5.3 - Alkaline lysis of bacteria for plasmid isolation

A single colony from an agar plate containing the plasmid to be isolated was inoculated into 400mL L-broth containing 100µg/mL ampicillin. For low-copy plasmids, 2mL 10% glucose was added to the culture to allow denser bacterial growth. Bacteria were then pelleted at 2190g for 15 minutes and the supernatant discarded. The bacterial pellet was resuspended in 8mL Buffer 1 (50mM glucose, 25mM Tris•HCl pH 7.4, 10mM EDTA pH 8), 80mg Lysozyme (Sigma) added, and the solution incubated for 5 minutes at room temperature. Sixteen mL of ice-cold

Buffer 2 (200mM NaOH, 1% SDS) was added and the solution incubated on ice for 10 minutes, followed by addition of 12mL ice-cold 5M potassium acetate and a further 10 minute incubation on ice. The solution was then centrifuged at 17,000g for 20 minutes at 4ºC. The supernatant was phenol/chloroform extracted and the DNA precipitated by addition of 0.6 volumes of isopropanol. Precipitated DNA was pelleted by centrifugation at 12,000g for 15 minutes. The pellet was washed with 1mL 70% ethanol, air-dried and resuspended in 1mL TE buffer.

2.5.4 - Caesium chloride density gradient plasmid purification

The DNA prepared by alkaline lysis had RNase A added to a final concentration of 100µg/mL and was incubated at 37ºC for 30 minutes. TE buffer (9mL) and 10.9g caesium chloride were added. Ethidium bromide (500µL of 10mg/mL stock) was then added and the sample loaded into a quick seal centrifuge tube, and centrifuged at 255,000g for 24-48 hours at 18ºC. Following centrifugation, bands containing supercoiled plasmid were removed by syringe and the ethidium bromide removed by serial extraction with equal volumes of MilliQ saturated butanol until the aqueous phase was clear. MilliQ water (2 volumes) was then added to the aqueous phase. Plasmid DNA was then ethanol/sodium acetate precipitated overnight at -20ºC. Precipitated DNA was pelleted at 12,000g for 15 minutes, washed with 70% ethanol, air-dried and resuspended in 400µL TE buffer. The DNA was then re-precipitated with sodium acetate and ethanol, washed and air-dried as above and resuspended in 200µL TE buffer. DNA was then quantitated on a NanoDrop 1000

Spectrophotometer (Thermo Scientific) and the concentration adjusted to 1mg/mL for use in transfections.

2.5.5 - Plasmid methylation

Plasmid was CpG methylated prior to transfection by treatment of 150µg plasmid with 200U *Sss*I methylase (New England Biolabs) and 640µM *S*-adenosylmethionine for 3 hours at 37ºC. Methylated plasmid was recovered by phenol-chloroform extraction and ethanol/sodium acetate precipitation.

2.5.6 – Plasmid information

Plasmid	Description	Purpose	Source
pGL3 Control	SV40 promoter and enhancer driving luciferase expression	Transient transfection for luciferase assay, positive control	Promega
pXP1-GM0.2	237bp of murine *GM-CSF* promoter (-200 to +37) in pXP1 vector	Transient transfection for luciferase assay	Dr. Peter Cockerill, ref. Osborne *et al*, 1995
pXP1GMWT	730bp of human *GM-CSF* promoter (-620 to +37) in pXP1 vector	Transient transfection for luciferase assay	Prof. Frances Shannon, ref. Cakouros *et al*, 2001
pXP1GMSp1m	As for pXP1GMWT, with promoter Sp1 site mutated	Transient transfection for luciferase assay	Prof. Frances Shannon, see Fig. 2.1 for specific mutation
pXP1GMCD28RRm	As for pXP1GMWT, with promoter CD28RR mutated	Transient transfection for luciferase assay	Prof. Frances Shannon, see Fig. 2.1 for specific mutation
pCDNA3.1	Contains neomycin resistance gene	Stable transfection, G418 resistance	Invitrogen
pBSXH10.5GMWT	10.5kb of human *GM-CSF* gene (enhancer, promoter and coding region)	Stable transfection	Prof. Frances Shannon, ref. Cakouros *et al*, 2001
pBSXH10.5GMSp1m	As for pBSXH10.5GMWT, with promoter Sp1 site mutated	Stable transfection	Prof. Frances Shannon, see Fig. 2.1 for specific mutation
pBSXH10.5GMCD28RRm	As for pBSXH10.5GMWT, with promoter CD28RR mutated	Stable transfection	Prof. Frances Shannon, see Fig. 2.1 for specific mutation

Table 2.5: Plasmids used in stable and transient transfection

The specific base substitutions in the Sp1m and CD28RRm mutant promoter variants are shown in Figure 2.1.

2.5.7 - Agarose gel electrophoresis

Nucleic acids were separated on a 1% or 2% (dependent on fragment size) agarose gel in TAE (40mM Tris acetate, 1mM EDTA) buffer at 100V for 60-75 minutes. The gel was incubated in TAE buffer containing 2μg/mL ethidium bromide for 15 minutes, rinsed, and bands visualised by ultraviolet illumination.

2.5.8 - Phenol/chloroform extraction

TE-saturated phenol (0.5 volumes) and chloroform (0.5 volumes) were added to the sample to be extracted in a Phase Lock Gel Light microcentrifuge tube (5 Prime) and shaken well. The sample was centrifuged at 13,000g for 5 minutes at room temperature, and the aqueous layer removed for DNA precipitation.

2.5.9 - Ethanol/sodium acetate precipitation

To precipitate DNA from solution, 0.1 volumes of sodium acetate (3M, pH 6.5) and 2.5 volumes of 100% ethanol were added to the sample and mixed well. The sample was then incubated overnight at -20ºC and the precipitated DNA pelleted at 13,000g for 15 minutes at room temperature. The DNA pellet was washed with 1mL 70% ethanol (13,000g, 5 minutes) and air-dried before resuspension in an appropriate volume of MilliQ water or TE buffer.

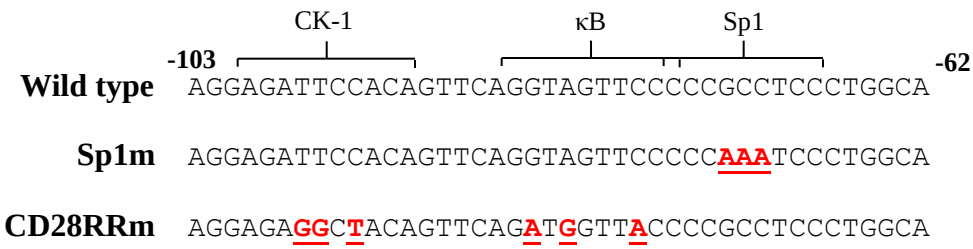

Figure 2.1: Promoter mutants used in transient and stable transfections. Three mutants of the human *GM-CSF* promoter from -103 to -62 are shown. On the wild type promoter, the NF-κB binding sites (CK-1 and κB) in the CD28RR and the Sp1 binding site are indicated. For the Sp1m and CD28RRm promoters, the relevant base substitutions that abolish the binding sites are indicated in red.

2.6 - Statistical analysis

All statistical analysis of data was performed using the GraphPad Prism® 5 program.

Chapter Three – The role of DNA methylation and the Sp1 transcription factor in regulating *GM-CSF* gene expression in T and B cells

3.1 - Introduction

It is known that for *GM-CSF* gene activation to occur, a number of factors must be present in the cell nucleus. The *GM-CSF* proximal promoter contains multiple transcription factor binding sites which have been implicated in the activation process (shown in Figure 1.1). Factors known to bind the promoter and regulate *GM-CSF* gene activation include NFAT (Shang *et al*, 1999) and Rel/NF-κB (Himes *et al*, 1996) family members, as well as Sp1 (Masuda *et al*, 1994), Ets, and AP-1 transcription factors (Wang *et al*, 1994). There are two different binding sites for Rel/NF-κB members, located adjacent to each other in the *GM-CSF* promoter, which have been particularly well characterised. These are a classical (i.e. p50/RelA dimer) NF-κB binding site and a variant site called the CK-1 element, which each bind dimers of different Rel/NF- κB family members.

Activation of the *GM-CSF* gene in response to immune signals requires changes to the chromatin structure of the *GM-CSF* promoter (Cockerill 1995; Cakouros *et al*, 2001), and this involves remodelling of a single nucleosome covering the promoter (Holloway *et al*, 2003). These remodelling events have different signal and transcription factor requirements to the subsequent transcription events. Chromatin remodelling at the promoter is dependent on the presence of Rel/NF-κB proteins in the nucleus, while the subsequent transcriptional events also require NFAT

(Brettingham-Moore *et al*, 2005). Along with the sets of transcription factors that are required for *GM-CSF* gene activation, the epigenetic environment of the *GM-CSF* promoter may also play a role in regulating gene activation in response to immune signals. The epigenetic environment of a gene is a combination of various factors, including the spatial organisation of nucleosomes on the gene, the distribution of histone modifications on these nucleosomes, and the methylation status of CpG dinucleotides in the DNA sequence (reviewed in Jaenisch and Bird, 2003). The contribution of epigenetic factors to regulation of the *GM-CSF* gene in response to immune signals and the differential response of the gene in different cell types is not well understood.

Although DNA methylation is a widely studied process in gene regulation, most studies have focused on methylation of CpG islands, which are regions of several hundred to several thousand base pairs in length containing a high density of CpG dinucleotides. CpG islands are often found close to or within gene promoters, and the methylation status of these CpG islands can determine the activity of the gene (reviewed in Sulewska *et al*, 2007). However, recent studies have identified cases where the methylation status of specific CpGs not associated with islands can also play an important role in regulating promoter activity. For example, Kitazawa and Kitazawa (2002) found that methylation of a single CpG in the receptor activator of NF-κB ligand (*RANKL*) gene promoter was important in repressing activation of the gene. Similarly, Kim *et al* (2007) found that demethylation of a specific CpG dinucleotide located in a DNase I hypersensitive site in the Th2 locus control region

correlated with *IL-4* expression. Furthermore, Bruniquel and Schwartz (2003) observed rapid demethylation of 6 specific CpG sites in the *IL-2* promoter upon gene activation in response to a stimulus in naïve T cells. In T cells that had previously been induced to express *IL-2*, this demethylated state was stably maintained. There are several CpG dinucleotides located in the vicinity of the *GM-CSF* promoter. While these do not constitute a CpG island, these recent findings raise the possibility that methylation of these CpG sites may play a role in regulating *GM-CSF* gene expression.

The aim of this chapter was to examine whether DNA methylation at the *GM-CSF* promoter is important in regulating the response of the gene to activating stimuli. To address this aim the expression of the *GM-CSF* gene in T and B cells, and the effect of DNA methylation on *GM-CSF* gene activation were examined.

3.2 - Results

The inducibility of the *GM-CSF* gene was examined in transformed T and B cell lines, as well as in primary T and B cell preparations. The activation of *GM-CSF* gene expression in T cells is known to involve the PKC and calcium signalling pathways, which can be pharmacologically induced via treatment with the phorbol ester PMA and calcium ionophore, respectively. EL-4 T cells as well as A20 and WEHI-231 B cells were left untreated (NS) or stimulated with PMA and ionophore (PI) for 4 hours. RNA was extracted, cDNA prepared, and *GM-CSF* mRNA expression analysed by qPCR. A substantial increase in *GM-CSF* mRNA levels upon PI stimulation was observed in EL-4 T cells, but not A20 or WEHI-231 B cells (Figure 3.1a). Similarly, PI stimulation resulted in increased expression of *GM-CSF* mRNA in primary mouse splenic $CD4^+$ T cells, but not in $CD19^+$ B cells (Figure 3.1b).

Since endogenous GM-CSF gene expression could not be induced in B cells, transient luciferase reporter assays were performed to determine if the *GM-CSF* promoter was capable of driving gene expression in these cells. A construct, pXP1-mGM0.2, containing 237bp of the *GM-CSF* promoter region coupled to a luciferase reporter gene (described in section 2.5.6) was transfected into EL-4 T cells and A20 B cells. After a 24h recovery period, the transfected cells were either left unstimulated or stimulated for 8h with PI, and protein isolated. Luciferase activity was then measured for both non-stimulated and PI stimulated cells. Cells were transfected in parallel with the pGL3 control plasmid (Promega), containing the

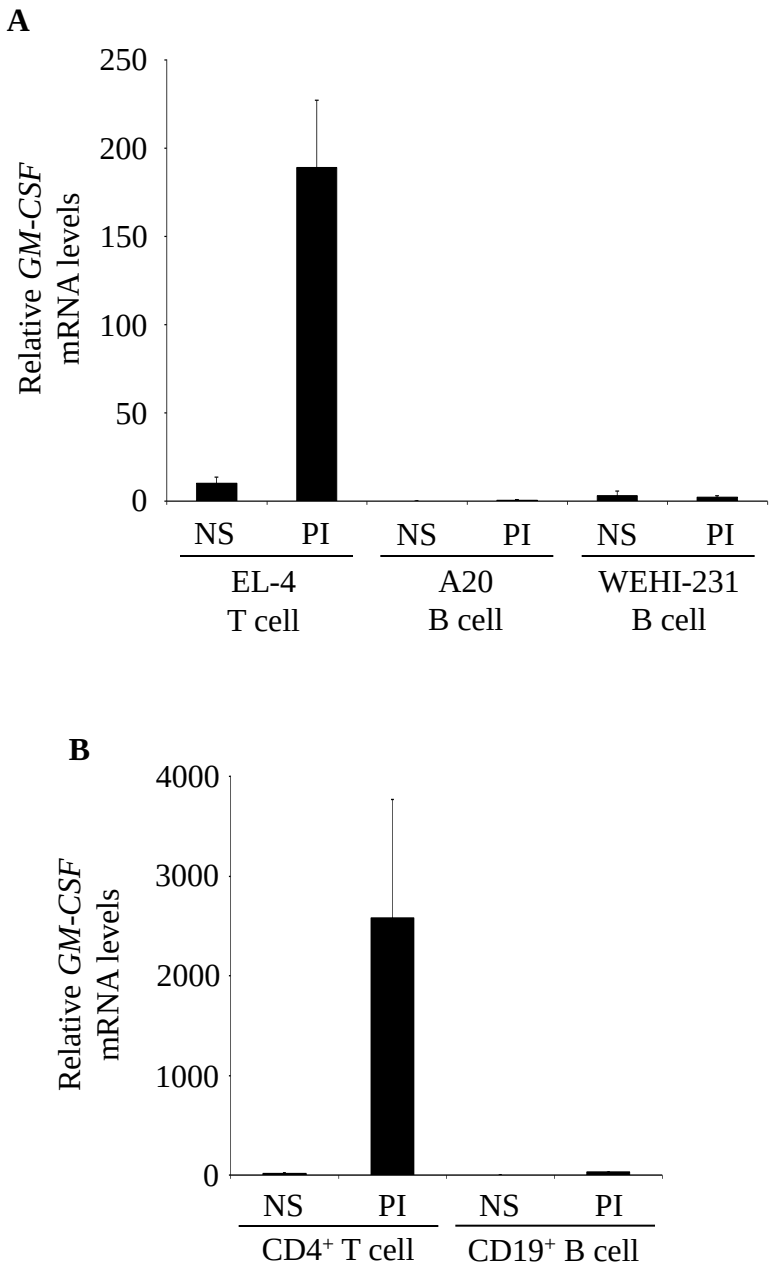

Figure 3.1: *GM-CSF* gene expression is inducible in T but not B cells. (A) *GM-CSF* mRNA levels were determined by RT-qPCR analysis of RNA extracted from EL-4 T cells, A20 B cells and WEHI-231 B cells either left untreated (NS) or treated for 4 hours with PMA and calcium ionophore (PI). Levels are shown relative to *GAPDH* mRNA expression. The mean and standard error of three independent experiments are shown. (B) GM-CSF gene expression was analysed as in (A) in primary mouse splenic $CD4^+$ T cells and $CD19^+$ B cells either untreated (NS) or treated with PI for 4 hours. The mean and standard error of two independent experiments are shown.

constitutively active simian virus 40 (*SV40*) promoter and enhancer coupled to the luciferase gene, to monitor transfection efficiency in the two cell lines. As shown in Figure 3.2a, there was no difference in luciferase activity between EL-4 T cells and A20 B cells when transfected with the pGL3 control plasmid, suggesting that the two cell lines transfect with equal efficiency. Luciferase activity driven by the *GM-CSF* promoter, both at a basal level and following PI treatment, was higher in the EL-4 T cells compared to the A20 B cells. However, an increase in luciferase activity was observed in the A20 B cells following stimulation, indicating that activation of the *GM-CSF* promoter is possible in these cells. Furthermore, the fold change in luciferase activity upon PI stimulation was comparable for the two cell lines (Figure 3.2a). Together these data suggest that, while endogenous *GM-CSF* gene expression cannot be induced in the B cell lines examined, the *GM-CSF* promoter can be activated in A20 B cells.

As noted in the introduction to this chapter, chromatin remodelling of a nucleosome covering the *GM-CSF* promoter is required for gene activation in response to immune signals. The reporter assay data presented in Figure 3.2a suggest that transcription from the *GM-CSF* promoter is possible in A20 B cells, so the block in *GM-CSF* activation in A20 B cells may be primarily at the chromatin remodelling level. In support of this, Brettingham-Moore *et al* (2008) found that chromatin remodelling events are blocked in A20 B cells. The Rel/NF-κB proteins RelA and c-Rel are required for chromatin remodelling of the *GM-CSF* promoter in EL-4 T cells (Brettingham-Moore *et al*, 2005), and therefore western blotting was performed

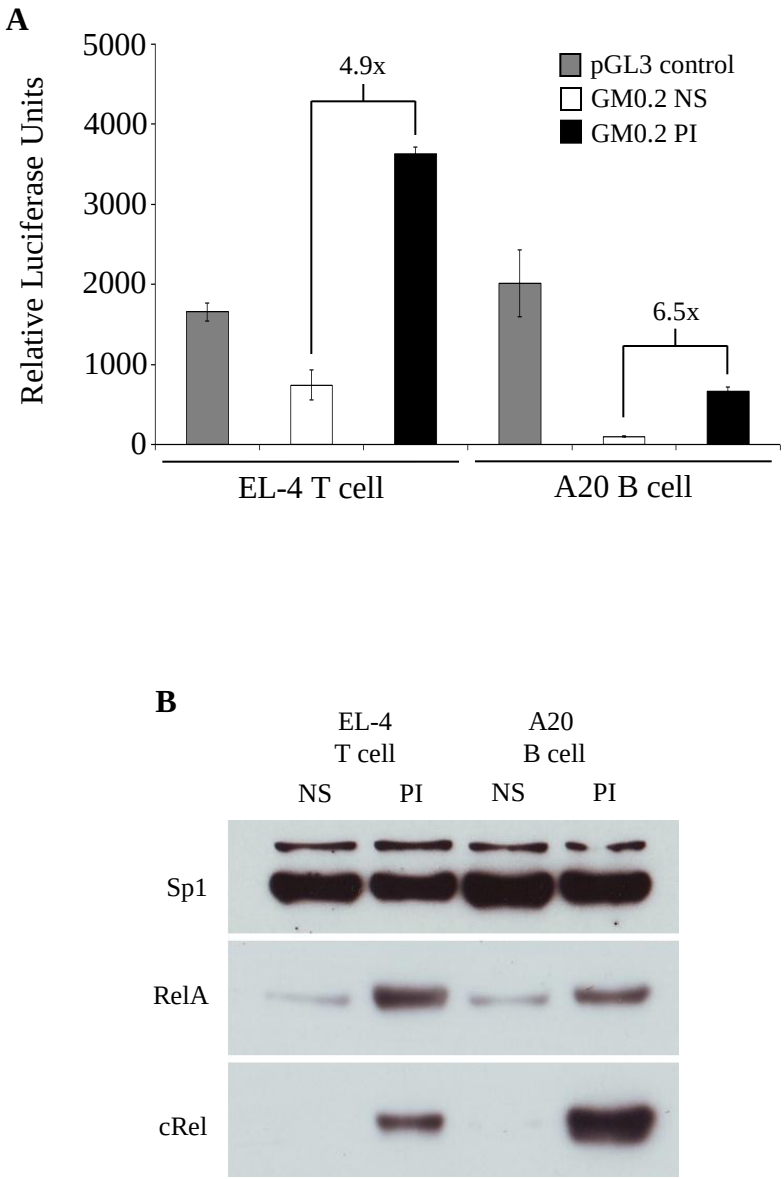

Figure 3.2: The *GM-CSF* promoter can be activated in A20 B cells. (A) The pGL3 luciferase control plasmid (5µg) and the pXP1-mGM0.2 luciferase reporter construct (GM0.2) containing the *GM-CSF* promoter (10µg) were transiently transfected into EL-4 T cells and A20 B cells. GM0.2 transfected cells were either left untreated (NS) or stimulated with PI for 8 hours. Protein was extracted from all cells, and luciferase activity measured. Fold changes between GM0.2 NS and PI are indicated. The mean and standard error of three independent experiments are shown. (B) Nuclear proteins were isolated from non-stimulated (NS) or 4 hour PI stimulated (PI) EL-4 T cells and A20 B cells and subjected to Western blotting with antibodies as indicated.

to determine the levels of these proteins in the A20 B cell nucleus. Nuclear proteins were extracted from non-stimulated and 4h PI stimulated EL-4 T cells and A20 B cells and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies for Sp1, RelA and cRel (Figure 3.2b). Little RelA or cRel was detected in the nuclei of unstimulated cells, but both proteins were detected in the nucleus of EL-4 T cells and A20 B cells upon PI stimulation. Equivalent levels of Sp1, which also has a binding site in the *GM-CSF* promoter, were observed in the nucleus in both cell types before and after stimulation, demonstrating relatively equal loading of proteins in each lane. Since the NF-κB proteins, which are required for chromatin remodelling at the *GM-CSF* promoter, are present in the A20 B cell nucleus following PI stimulation, other factors that may explain the differential response of the *GM-CSF* gene to stimulation between T and B cells were investigated. As activation of a reporter plasmid, but not the endogenous *GM-CSF* gene, could be achieved in B cells, factors known to influence chromatin environment were examined.

Methylation of CpG dinucleotides has been implicated in the regulation of many genes. The *GM-CSF* gene does not contain a CpG island; however, the region encompassing the promoter contains several CpG dinucleotides that are potential methylation sites. A diagram of these sites and their locations in relation to the *GM-CSF* promoter is shown in Figure 3.3a. One site, referred to here as CpG +1, is located approximately 100bp into the transcribed region of *GM-CSF*. The CpG -1 site is located within the proximal promoter itself, forming part of the Sp1 binding

site. Sites CpG -2 and CpG -3 are located approximately 200bp and 300bp respectively upstream of the TSS. Finally, one of several CpG sites in the *GM-CSF* enhancer, located 2kb upstream of the promoter, was also examined. All of these CpG sites are also located in recognition sites for the *Aci*I restriction enzyme. This enzyme has the recognition site 5' C|CGC 3', however, when the CpG dinucleotide in this site is methylated, the enzyme is unable to cut. To determine the methylation status of these CpGs in EL-4 T cells, A20 B cells and WEHI-231 B cells, genomic DNA was isolated and digested with *Aci*I. Undigested and *Aci*I digested DNA was amplified by qPCR with primer sets spanning the sites of interest. The ratio of product obtained from digested versus undigested DNA was determined to calculate the percentage of DNA methylation at each site; strictly speaking, the percentage of DNA in each sample in which the site examined was methylated (Figure 3.3b). Across the 5 sites assessed, there were clear differences in DNA methylation between the cell lines. At the enhancer site, EL-4 T cells and WEHI-231 B cells exhibited complete methylation, while in A20 B cells this site showed only approximately 70% methylation. Likewise, CpG -3 was completely methylated in EL-4 and WEHI-231 cells, but only approximately 20% methylated in A20 cells. Sites -2, -1 and +1 were all fully methylated in both B cell lines, but displayed progressively lower levels of methylation in EL-4 T cells, with levels approximately 90%, 40% and 10% respectively. Since CpGs -1 and +1 displayed the greatest levels of variation between the *GM-CSF* expressing T cells and the non-expressing B cell lines, and are located in the promoter and transcribed regions of the gene, respectively, these sites were also examined in primary mouse splenic T and B cells (Fig 3.3c). Interestingly,

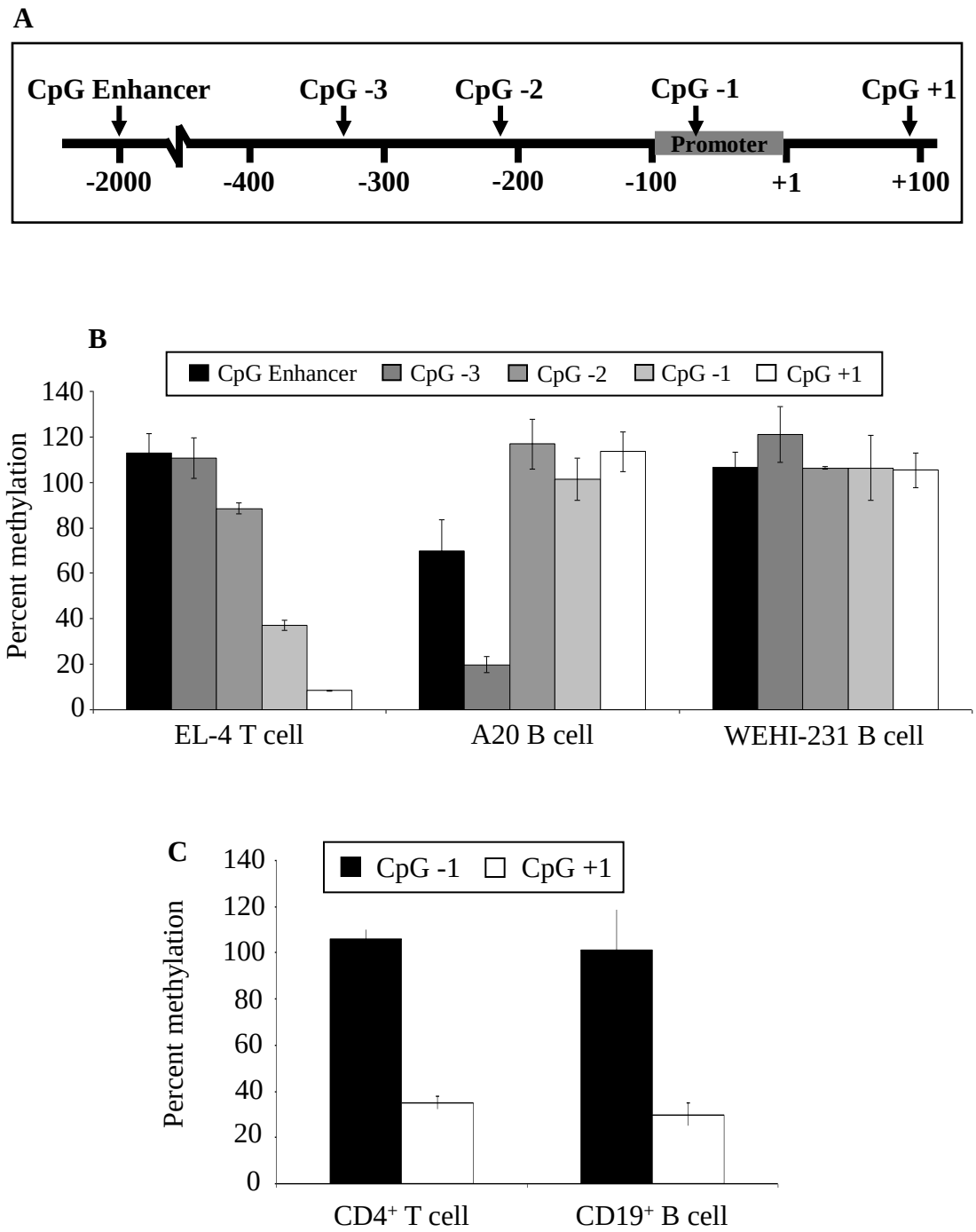

Figure 3.3: Differential patterns of DNA methylation across the *GM-CSF* gene exist in cell lines, but not primary cells. (A) Schematic map of CpG sites to be investigated in the *GM-CSF* gene. (B) Genomic DNA from EL-4 T cells, A20 B cells and WEHI-231 B cells was digested with the methylation-sensitive restriction enzyme *Aci*I. qPCR was performed on digested and undigested DNA using primer sets covering the methylation sites shown in (A), and the percentage methylation at each site calculated. The mean and standard error of three independent experiments are shown. (C) The methylation assay was performed on genomic DNA isolated from primary mouse $CD4^+$ T cells and $CD19^+$ B cells, as outlined in (B). The mean and standard error of two independent experiments are shown.

the variation seen in the transformed cell lines was not replicated in primary cells. Rather, CpG -1 was 100% methylated in both $CD4^+$ T cells and $CD19^+$ B cells, while CpG+1 was approximately 30% methylated in both cell types.

While this implies that differences in methylation are unlikely to contribute to the differential expression of the *GM-CSF* gene in primary T and B cells, they may contribute to the differential regulation observed in cell lines. To investigate this possibility the effect of methylation on promoter activity was examined. The pXP1-mGM0.2 promoter luciferase construct was methylated with the *Sss*I CpG methylase enzyme. To confirm that methylation was complete, methylated and non-methylated plasmid were digested with *Aci*I, and separated by agarose gel electrophoresis alongside undigested controls (Figure 3.4a). While the unmethylated plasmid was digested by *Aci*I, following incubation with *Aci*I the *Sss*I-methylated plasmid showed the same digestion pattern as the undigested controls. These data indicate that the plasmid had undergone complete CpG methylation, preventing *Aci*I restriction activity. Methylated and unmethylated plasmids were transfected into EL4 T cells, which were either left untreated (NS) or stimulated with PI for 8 hours. Protein was extracted and luciferase activity was assessed. While the unmethylated plasmid exhibited basal activity, which increased upon stimulation, the *Sss*I-methylated plasmid displayed very low levels of activity, even following stimulation (Figure 3.4b). This indicates that the DNA methylation status of the promoter may play a role in regulating *GM-CSF* promoter activity.

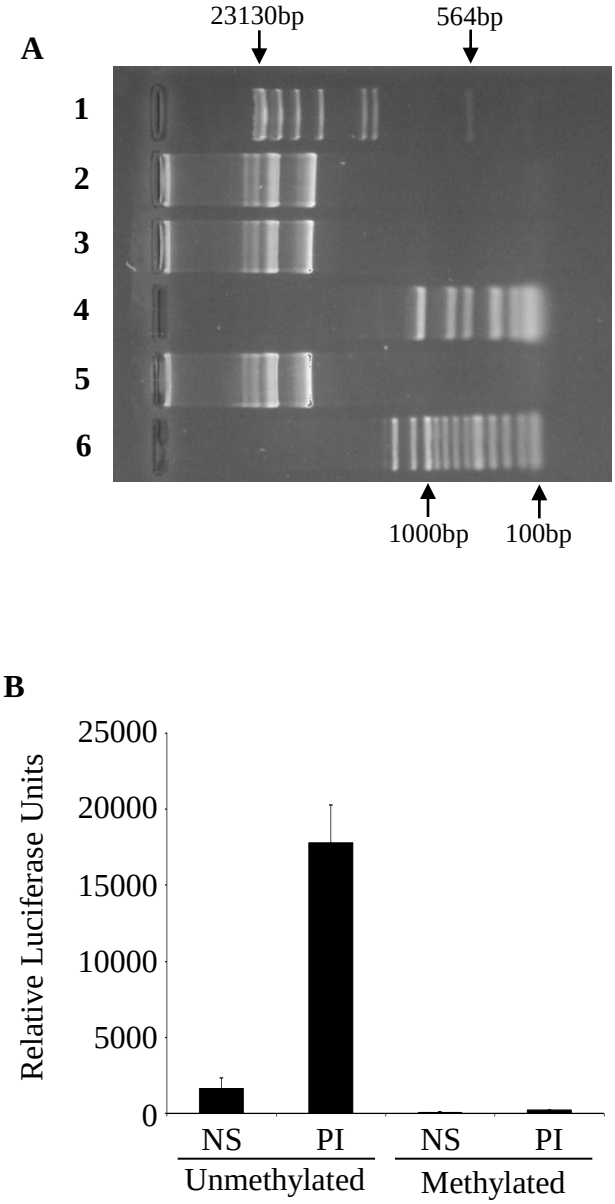

Figure 3.4: DNA methylation affects transcription from a *GM-CSF* reporter plasmid. (A) The pXP1-mGM0.2 plasmid was either treated with *Sss*I methylase or left untreated, digested with *Aci*I or left undigested, and analysed by agarose gel electrophoresis. Lanes contain (1) *Hind*III-digested Lambda DNA size marker; (2) non-*Sss*I treated, non-*Aci*I digested plasmid; (3) *Sss*I treated, non-*Aci*I digested plasmid; (4) non-*Sss*I treated, *Aci*I digested plasmid; (5) *Sss*I treated, *Aci*I digested plasmid and (6) 100bp size marker. Sizes of selected marker bands are indicated. (B) Control (unmethylated) and *Sss*I-treated (methylated) pXP1-mGM0.2 plasmid was transfected into EL-4 T cells. Cells were untreated (NS) or stimulated with PI for 8h (PI), total protein extracted and luciferase activity measured. The mean and standard error of two independent experiments are shown.

To further test the role of DNA methylation in regulating *GM-CSF* gene expression, the effect of 5-aza-2-deoxycytidine (azacytidine), an inhibitor of DNA methyltransferase, on DNA methylation and *GM-CSF* gene expression was studied. EL-4 T cells and A20 B cells were treated with azacytidine for 24 hours, at which point the azacytidine was removed and the cells incubated for a further 24 hours. Genomic DNA was extracted and analysed for DNA methylation status as in Figure 3.3b. At CpG -1, within the promoter, it was found that azacytidine treatment significantly decreased DNA methylation in both EL-4 T cells (from approx. 50% to approx. 20%) and A20 B cells (approx. 100% to approx. 40%) (Figure 3.5a; $P<0.05$, unpaired t-test). To examine *GM-CSF* gene expression, azacytidine treated cells were either left untreated (NS) or stimulated with PI for 4 hours, and *GM-CSF* mRNA levels measured. In EL-4 T cells, in the absence of treatment with azacytidine an increase in *GM-CSF* mRNA expression upon stimulation was observed, as seen previously in Figure 3.1a. Pretreatment with azacytidine resulted in a significantly greater increase in *GM-CSF* mRNA expression upon stimulation than without azacytidine pretreatment (Figure 3.5b; $P<0.05$, unpaired t-test). In A20 B cells, however, no increase in *GM-CSF* gene expression upon stimulation was observed, in either the untreated or azacytidine treated cells (Figure 3.5b). This suggests that while DNA demethylation can result in increased *GM-CSF* gene expression, as seen in EL-4 T cells, it is not able to facilitate *GM-CSF* gene activation in A20 B cells.

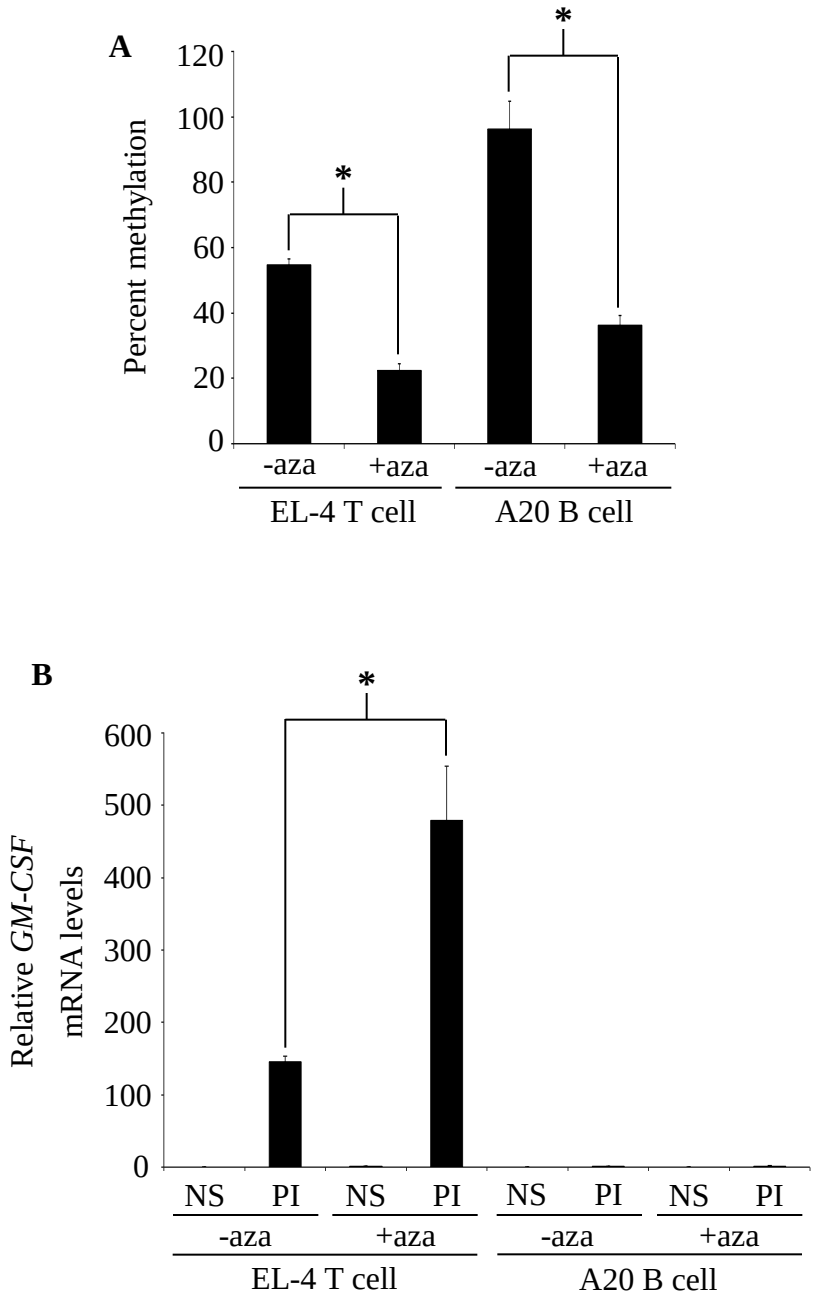

Figure 3.5: DNA demethylation is not sufficient to facilitate *GM-CSF* gene expression in A20 B cells. (A) EL-4 T cells and A20 B cells were either left untreated (NS) or treated with 5-azacytidine (aza) for 24h, then cultured for a further 24h. Genomic DNA was isolated and methylation at CpG -1 examined by the *Aci*I based methylation assay. The mean and standard error of three independent experiments are shown. *P<0.05, unpaired t-test. (B) EL-4 T cells and A20 B cells were either left untreated or treated with aza as in (A), then were either left unstimulated (NS) or stimulated with PI for 4h. RNA was extracted and *GM-CSF* mRNA levels assessed using RT-qPCR. The mean and standard error of three independent experiments are shown. *P<0.05, unpaired t-test.

As previously mentioned, the CpG -1 site is located within an Sp1 binding site in the *GM-CSF* promoter. It is therefore possible that changes in the methylation status of this CpG dinucleotide may alter transcriptional activation of the *GM-CSF* promoter, through effects on Sp1 binding. As Sp1 was found to be constitutively present in the nuclei of both EL-4 T cells and A20 B cells (Figure 3.2b), recruitment of the Sp1 transcription factor to the *GM-CSF* promoter in these cell lines was examined using chromatin immunoprecipitation (ChIP). DNA was crosslinked to proteins with formaldehyde, sheared by sonication and immunoprecipitated with an Sp1 antibody. The precipitated DNA was then analysed by qPCR to determine the levels of Sp1 binding to the *GM-CSF* promoter. Relative to EL-4 T cells, A20 B cells had significantly lower levels of Sp1 bound at the *GM-CSF* promoter (Figure 3.6; $P<0.05$, one-sample t-test). Analysis using primers targeted to a region 1.1kb upstream of the promoter as a control (see section 2.2.3) showed negligible Sp1 binding at this region in either cell type, suggesting that Sp1 binding to the promoter region is specific.

Since Sp1 was enriched at the *GM-CSF* promoter in T compared to B cell lines, the role of Sp1 in *GM-CSF* promoter regulation was examined further. The effect of abolishing the Sp1 binding site on gene transcription was examined in reporter assays. Luciferase reporter plasmids, consisting of the pXP1 plasmid containing 730bp of the human *GM-CSF* promoter (see section 2.5.6), were transfected into EL4 T cells, which were then either left untreated (NS) or stimulated with PI for 8

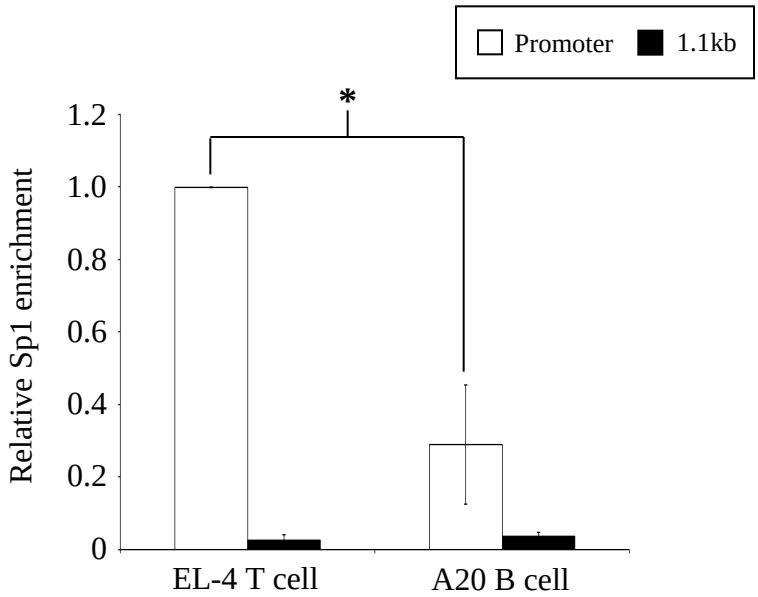

Figure 3.6: Sp1 is enriched at the *GM-CSF* promoter in EL-4 T cells. Genomic DNA crosslinked to Sp1 was isolated from EL-4 T cells and A20 B cells by ChIP and analysed by qPCR using primers that amplify a region of the *GM-CSF* promoter and a region 1.1kb upstream of the promoter (used as a control). Data is graphed relative to the promoter region in EL-4 T cells. The mean and standard error of five independent experiments are shown. *P<0.05, one-sample t-test**.**

hours and assessed for luciferase activity. The plasmids used contained either a wild type (WT) *GM-CSF* promoter, or one of two mutant *GM-CSF* promoters, abolishing either the Sp1 binding site (Sp1m) in the promoter or the two Rel/NF-κB binding sites in the CD28RR of the promoter (CD28RRm). The CD28RRm was included in these experiments as the Rel/NF-κB sites are known to be important in both chromatin remodelling and transcriptional activation of the *GM-CSF* promoter (Holloway *et al*, 2003; Brettingham-Moore *et al*, 2005). The binding sites present in each promoter construct are shown schematically in Figure 3.7a. In EL-4 T cells the WT promoter was activated by PI treatment (Figure 3.7b), to a level similar to that seen previously for the mouse promoter (see Figure 3.2a). The Sp1m promoter, however, exhibited both lower basal activity and no response to PI stimulation. The CD28RRm promoter displayed basal activity levels in unstimulated cells similar to the WT promoter, but with reduced activation in response to PI stimulation (Figure 3.7b). These data suggest that both the Sp1 and Rel/NF-κB binding sites are important for transcriptional activation of the *GM-CSF* promoter.

These mutants were then also tested as stable transgenes in EL-4 T cells. Plasmids containing a 10.5kb fragment consisting of the enhancer, promoter and full coding region of the human *GM-CSF* gene (described in section 2.5.6) were stably integrated into the genome of EL-4 T cells, together with a plasmid encoding a neomycin resistance gene to allow for selection. Stably transfected pools were generated containing the wild type plasmid and the two mutant promoter plasmids (WT, Sp1m, CD28RRm) shown in Figure 3.7a. The average number of copies

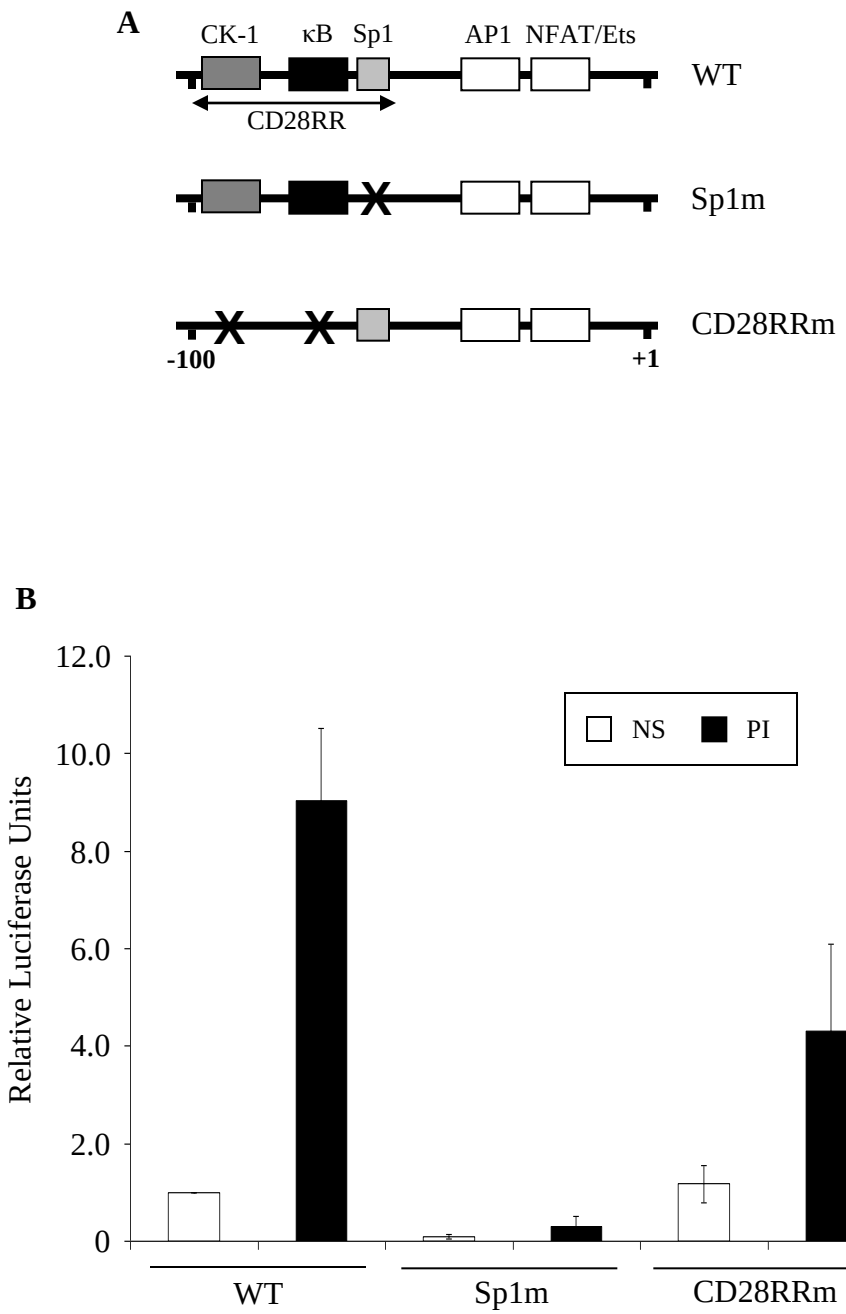

Figure 3.7: Mutating Sp1 and NF-κB transcription factor binding sites in the *GM-CSF* promoter affects promoter activity. (A) Schematic showing the transcription factor binding sites present in the WT, Sp1m and CD28RRm *GM-CSF* promoter reporter plasmids. (B) Luciferase reporter plasmid constructs containing the three *GM-CSF* promoter mutants in the pXP1 vector were transfected into EL-4 T cells and luciferase activity with (PI) or without (NS) an 8 hour PI stimulation was assessed. Luciferase activity is graphed relative to wild-type NS. The mean and standard error of two independent experiments are shown.

incorporated in each cell pool was determined by qPCR using primers that distinguished between the human and endogenous mouse *GM-CSF* promoters (Figure 3.8a; primers described in section 2.2.3). The stable pools were then either left untreated (NS) or stimulated with PI for 4 hours, and RNA extracted for cDNA synthesis and analysis by qPCR. Levels of the h*GM-CSF* mRNA generated from each transgene were measured, normalised to the expression of the *GAPDH* housekeeping gene, and then further normalised to the transgene copy number. Expression from the WT transgene tended to increase upon PI stimulation. The Sp1 mutant showed similar basal gene expression to WT, but with a marked increase in expression upon stimulation. The CD28RR mutant displayed lower basal gene expression than the WT or Sp1m transgenes, and did not display any increase in expression following stimulation (Figure 3.8b). Expression of the endogenous mouse *GM-CSF* gene was the same between all stable lines (data not shown). Figure 3.8c represents the same data as Figure 3.8b, graphed instead as the fold change between NS and PI samples for each stable pool. This data clearly shows that the WT gene is activated by PI stimulation, the Sp1 mutant is more inducible than the WT and that the CD28RR mutation abrogates the response of the gene to PI stimulation. Put together these data suggest that the Sp1 binding site containing the CpG dinucleotide in the *GM-CSF* promoter is important in regulation of the *GM-CSF* gene, although mutation of this site has different effects in a chromatin-free context compared to when it is incorporated into cells as a transgene.

A

Stable pool	Copies transgene/cell
WT	6.0
Sp1m	9.0
CD28RRm	1.7

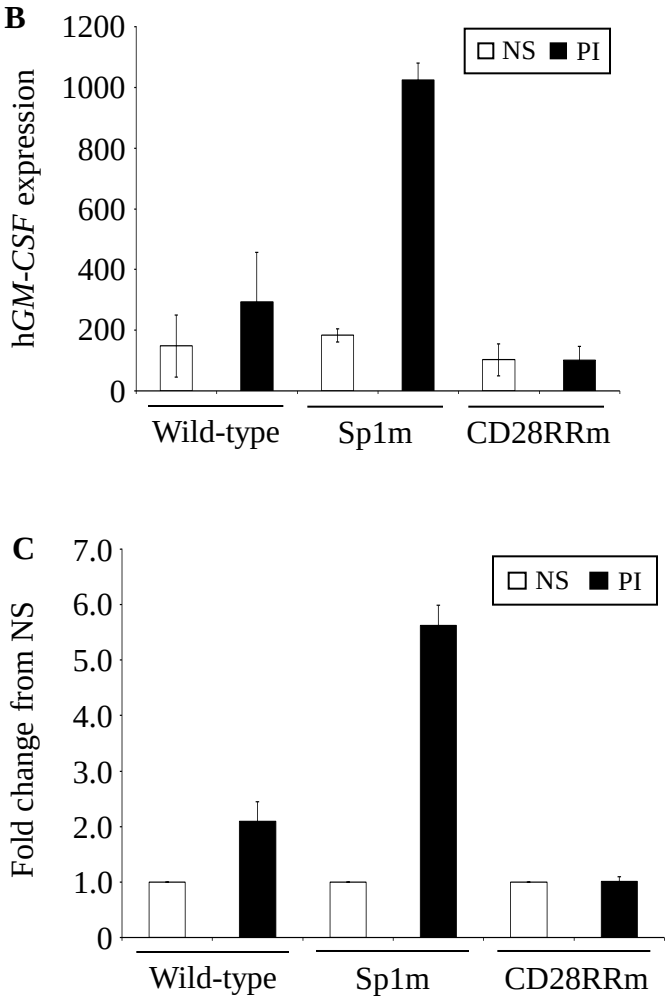

Figure 3.8: Mutation of the Sp1 and NF-κB binding sites affects *GM-CSF* gene transcription in stably transfected cell lines. 10.5kb fragments of the human *GM-CSF* gene containing the wild type, Sp1m or CD28RRm promoter mutants were stably integrated as transgenes into EL-4 T cells. (A) The average number of transgenes incorporated per cell was calculated for each stable pool. (B) Cells were treated with PI for 4 hours, RNA isolated, and RT-qPCR performed to assess expression of the transgene (shown adjusted to relative transgene copy number and GAPDH). The mean and standard error of two independent experiments are shown. (C) The fold change in mRNA expression for PI samples compared to NS samples was calculated for each stable pool. The mean and standard error of two independent experiments are shown.

3.3 - Discussion

GM-CSF gene expression is inducible in T cells, but not in B cells (Figure 3.1). Furthermore, previous work from this laboratory has demonstrated that chromatin remodelling events at the *GM-CSF* promoter, required for activation of the gene in T cells, is blocked in B cells (Brettingham-Moore *et al*, 2008). The data presented in this chapter demonstrate that this is not due to differences in the presence of the Sp1 and Rel/NF-κB transcription factors that are involved in activation of the *GM-CSF* gene (Figure 3.2b) (Kochetkova *et al*, 1997; Cakouros *et al*, 2001). While it is possible that differences in other transcription factors required for transcriptional activation exist in B compared to T cells, examination of the *GM-CSF* promoter in a transiently transfected luciferase reporter plasmid, in which the normal chromatin environment of the gene is absent (Jeong and Stein, 1994), showed that the *GM-CSF* promoter is capable of driving transcription in B cells (Figure 3.2a). This further suggests that repression of *GM-CSF* gene activation in B cells is not due to a lack of the transcription factors required for its transcriptional activation. This implies that the ability of the *GM-CSF* gene to respond to inducing signals is governed at least in part by epigenetic factors, in addition to the transcription factors known to regulate this gene.

Examination of the DNA methylation status of the *GM-CSF* promoter revealed differences between EL-4 T cells, A20 B cells and WEHI-231 B cells (Figure 3.3b). An obvious difference between the T cell line compared to the two B cell lines was the methylation status of two CpG dinucleotides, -1 and +1, located within the *GM-*

CSF gene promoter and the transcribed region of the gene respectively. EL-4 T cells displayed low levels of methylation at these sites, while they were completely methylated in both B cell lines. Treatment of EL-4 T cells and A20 B cells with azacytidine was able to reduce the levels of DNA methylation, as assayed at site -1. While this DNA demethylation increased *GM-CSF* gene expression in response to PI stimulation in EL-4 T cells, it did not affect gene expression in A20 B cells (Figure 3.5). Previous work has shown that chromatin remodelling events at the *GM-CSF* promoter are blocked in B cells compared to T cells (Brettingham-Moore *et al*, 2008), and therefore DNA methylation may be affecting gene activation at a step subsequent to chromatin remodelling. In support of this, CpG methylation of a reporter plasmid containing the *GM-CSF* promoter dramatically reduced transcriptional activation of the promoter (Figure 3.4). While it is possible that the effects seen on the reporter are at least partially due to methylation of CpGs in the plasmid backbone, these data suggest that DNA methylation is acting to regulate gene expression at the transcriptional level. Potentially, if chromatin remodelling of the *GM-CSF* promoter could be induced in B cells, the effect of DNA demethylation of the promoter may become apparent.

The CpG dinucleotide in the *GM-CSF* promoter is situated in an Sp1 binding site. Given that the data presented here suggests that DNA methylation status may affect transcriptional activation of the promoter, it is possible that this is due to effects on the binding of the Sp1 transcription factor to this site. The Sp1 binding site also overlaps the CD28RR of the promoter, which contains two Rel/NF-κB family

member binding sites. The importance of these sites is well characterised (Masuda *et al*, 1994; Thomas *et al*, 1997), although due to the overlap between the CD28RR and the Sp1 binding site, previous studies have had difficulty distinguishing the exact contribution of each binding motif to *GM-CSF* gene regulation (Cakouros *et al*, 2001; Kochetkova *et al*, 1997). Sp1 is constitutively present in the cell nuclei of EL-4 T cells and A20 B cells at approximately equal levels. Despite this, Sp1 is enriched at the *GM-CSF* promoter in EL-4 T cells compared to A20 B cells (Figure 3.6), in which the Sp1 site is highly methylated. It is therefore possible that differential Sp1 binding, due to differential methylation, may contribute to the disparate responses of the *GM-CSF* gene between T and B cells. In support of this, previous studies have demonstrated an effect of DNA methylation on Sp1 binding. DNA methylation reduced binding of Sp1 to the dihydropyrimidine dehydrogenase (*DYPD*) gene promoter (Zhang et al 2007) and the citrate carrier (*CIC*) gene promoter (Iacobazzi *et al*, 2008). In contrast, it did not appear to influence binding of Sp1 to its cognate site in the multi-drug resistance 1 (*MDR1*) gene promoter (Kusaba *et al*, 1999). Alternatively, it is possible that the increased Sp1 binding in T cells blocks DNA methylation at the promoter, as previous studies have demonstrated a role for Sp1 in preventing DNA methylation of regulatory elements (Brandeis *et al*, 1994; Macleod *et al*, 1994)

The role of Sp1 in *GM-CSF* gene activation was further examined by use of transient and stably transfected promoter mutants. In the transient expression studies, mutating the two Rel/NF-κB binding sites in the CD28RR moderately affected

promoter activation compared to the wild type promoter. However, mutating the Sp1 binding site resulted in a decrease in both basal and stimulated promoter activity (Figure 3.7). Interestingly, when activity of the mutant promoters was examined in the stable context, the results were markedly different. In this case the Sp1 mutant was more inducible that the WT construct, while the CD28RRm was not inducible (Figure 3.8). The CD28RRm results conform to our previous understanding of *GM-CSF* gene regulation. It has been demonstrated previously that Rel/NF-κB proteins are required for remodelling of the promoter nucleosome, as well as subsequent transcriptional activation (Brettingham-Moore *et al*, 2005). Therefore the effects of this mutation are seen most dramatically in a chromatin context in the stably transfected cells, as presumably chromatin remodelling events required for gene activation do not occur at the mutant promoter. This is in keeping with previous mutational analysis of the *GM-CSF* promoter undertaken by Cakouros *et al* (2001). A "2CK-1" mutation, in which the classical NF-κB site in the *GM-CSF* promoter was mutated into a CK-1 site, was found to drive activity of a transiently transfected promoter reporter equivalently to the WT promoter. However, when examined as a stable transgene, the 2CK-1 mutant was unable to drive *GM-CSF* expression due to a lack of chromatin remodelling in response to PI stimulus.

The effect of mutating the Sp1 binding site is more complicated. In the transient model, the mutation drastically reduces activation of the *GM-CSF* promoter, implying that it is an important factor in transcriptional activation of the promoter. However, in the stable model, the mutation has the opposite effect, with gene activity

upon stimulation increasing above that shown by the WT gene. Hyperactivity of the *GM-CSF* promoter in a stable, but not transient context has been observed previously. Cakouros *et al* (2001) found that a "2κB" mutation, in which the CK-1 site was mutated to a classical NF-κB site, was hyperactive in a stable cell line model, but had no effect in transient transfections. It has previously been shown that mutation of the Sp1 site affects NF-κB binding, and vice versa (Kaushansky *et al*, 1996; Kochetkova *et al*, 1997; Cakouros *et al*, 2001) and the factors may therefore be acting co-operatively to influence the local chromatin environment. Such co-operative regulation of chromatin environment by these factors has been demonstrated previously at the human immunodeficiency virus (HIV) long terminal repeat (Pazin *et al*, 1996; Widlak *et al*, 1997). Further studies to investigate binding of Sp1 and NF-κB to the promoter mutant and examine the chromatin environment of the promoter would shed light on this possibility. Another possible explanation for these disparate effects is that the plasmids used in the transient luciferase assay only contain 730bp encompassing the *GM-CSF* promoter, whereas the plasmid used for the stable transfections contains the 10.5kb full-length *GM-CSF* gene, which includes an upstream enhancer region. Promoters and enhancers are known to interact in activating gene transcription (reviewed in West and Fraser, 2005), and the presence of the enhancer in the stably transfected model may be contributing to the observed differences in Sp1m activity.

The data obtained from the cell line analysis in this study suggest that Sp1 binding plays an important role in regulation of the *GM-CSF* promoter. In addition it has

shown that DNA methylation can contribute to the regulation of the *GM-CSF* promoter, and is consistent with it acting to affect the transcriptional activation of the promoter. However, while differences in DNA methylation were observed at the *GM-CSF* promoter in the transformed T and B cell lines, no such differences were observed when the same sites were surveyed in primary mouse splenic $CD4^+$ T cells and $CD19^+$ B cells. Rather, the CpG sites displayed the same levels of methylation in each cell type (Figure 3.3c). However, epigenetic mechanisms of gene regulation can be perturbed in transformed cells (reviewed in Jones and Baylin, 2007). For example, a study of primary and transformed prostate epithelial cells showed that genes can undergo an epigenetic "switch" from Polycomb mediated repression in primary cells to a more stable form of DNA methylation mediated repression in transformed cells (Gal-Yam *et al*, 2008). Hence, it may be possible that in the primary cells, DNA methylation is not the dominant form of repression, with other factors playing a more important role. Given that differences in DNA methylation do not explain the differential inducibility of the *GM-CSF* gene in primary T and B cells, other epigenetic factors may be responsible, and warrant further investigation.

Chapter Four – Chromatin modifications and chromatin remodelling proteins at the *GM-CSF* promoter

4.1 - Introduction

In the previous chapter, *GM-CSF* gene expression was demonstrated to be inducible in T cells, but not B cells. It was hypothesised that this differential response of the gene to stimulation was due to differences in the epigenetic environment of the gene, rendering it permissive to transcription in T cells but unresponsive in B cells. In addition to DNA methylation, which was examined in Chapter 3, modification of chromatin structure plays an important role in the regulation of gene expression. Two general mechanisms serve to alter chromatin structure. Firstly, covalent modification of histone tail residues can either directly disrupt histone-histone interactions, affecting higher order structure, or provide recognisable signals for the recruitment of transcription factors and chromatin remodelling complexes (CRCs) (reviewed in Berger, 2007). Secondly, CRCs can directly alter nucleosome positioning and hence chromatin structure by utilising ATP to facilitate the sliding or displacement of individual nucleosomes (reviewed in Gangaraju and Bartholomew, 2007).

Histone modifications, and their involvement in gene regulation, have been extensively studied. Acetylation of histone lysine residues is widely regarded as a mark of transcriptionally active genes (reviewed in Selvi and Kundu, 2009). In support of this, genome-wide analyses of chromatin modifications have shown

enrichment of histone H3 and H4 acetylation at many active gene promoters (Schübeler *et al*, 2004; Roh *et al*, 2005). Histone methylation can also influence the activity of a gene, either positively or negatively depending on the specific modification. Schübeler *et al* (2004) found that in addition to histone acetylation, methylation of lysines 4 and 79 of histone H3 was also associated with active gene promoters. The trimethylation of histone H3 lysine 27 (H3K27me3), however, is generally associated with the promoters of silenced genes (Cao *et al*, 2002).

While these studies have focused on constitutively active or silent genes, there is also now some information on the role of histone modifications in the regulation of inducible gene transcription. A genome-wide analysis performed in human T cells discovered the expected pattern of enriched H3 acetylation and H3K4me3 at active gene promoters, and enriched H3K27me3 at silent promoters. However, genes that were not expressed in resting cells but rapidly inducible upon stimulation also showed high levels of H3 acetylation and H3K4me3 at their promoters (Roh *et al*, 2006). A study of three Wnt-inducible genes in myogenic, neural and embryonic stem cell lines had similar findings. Analysis of these genes revealed that in cell types where the genes were inducible, the gene promoters displayed low levels of DNA methylation and H3K27me3, and enriched levels of histone H3 acetylation. In cell types where the genes were not inducible, the opposite pattern of epigenetic modifications was observed (Wöhrle *et al*, 2007). Similarly, Donati *et al* (2006) examined a set of endoplasmic reticulum (ER) stress induced genes and showed that the inducible genes had high levels of histone H3 acetylation and H3K4 and H3K79

methylation at their promoters. Therefore it appears that the distribution of histone modifications may mark genes for inducible activation. In the case of the ER inducible genes, the nucleosomes marked by hyper-acetylation were depleted upon gene activation.

Activation of the *GM-CSF* gene is known to involve similar chromatin remodelling events. A single nucleosome positioned over the promoter is remodelled upon gene activation (Holloway *et al*, 2003) and this has been shown to involve histone loss (Chen *et al*, 2005). Similar mechanisms may therefore exist to mark the *GM-CSF* promoter for activation in T cells. In support of this, the *GM-CSF* promoter chromatin was found to be hyperacetylated compared to other regions of the gene in T cells. Furthermore, the Brg1 protein, a core ATPase component of the SWI/SNF chromatin remodelling complex, is preferentially enriched at the *GM-CSF* promoter in EL-4 T cells compared to A20 B cells (Brettingham-Moore *et al*, 2008), and is required for chromatin remodelling in EL-4 T cells, suggesting that the SWI/SNF complex is involved in *GM-CSF* activation.

It is possible that these epigenetic factors mark the *GM-CSF* gene for activation in T cells. The aims of this chapter were therefore to examine the distribution of chromatin modifications and chromatin remodelling complexes at the *GM-CSF* promoter between T and B cells, and determine the role that these epigenetic factors play in regulating *GM-CSF* gene response.

4.2 - Results

As histone acetylation has been well characterized as a mark of active or transcriptionally permissive genes, and H3K9 acetylation has previously been found to be enriched at the *GM-CSF* promoter compared to other regions of the gene, in EL-4 T cells (Brettingham-Moore *et al*, 2008), the level of histone H3 acetylation at the *GM-CSF* promoter was measured using chromatin immunoprecipitation in EL-4 T cells (that express *GM-CSF*) and A20 and WEHI-231 B cells (which do not, as shown in Figure 3.1a). In both A20 and WEHI-231 B cells, levels of promoter H3 acetylation were approximately half that observed at the promoter in EL-4 T cells (Figure 4.1a). A similar pattern was observed between primary $CD4^+$ T cells and $CD19^+$ B cells, with T cells again displaying approximately twice the level of acetylation at the *GM-CSF* promoter compared to B cells (Figure 4.1b).

To determine whether this difference in acetylation is likely to influence the activity of the *GM-CSF* gene in these cell types, EL-4 T cells and A20 and WEHI-231 B cells were treated with the histone deacetylase inhibitor Trichostatin A and the effect on histone acetylation at the promoter and *GM-CSF* gene expression monitored. Firstly, the effect of TSA treatment on promoter histone H3 acetylation was examined. Following treatment with TSA, a large increase in promoter H3 acetylation was detected in EL-4 T cells. In both A20 and WEHI-231 B cells, significant increases in promoter H3 acetylation were also observed, reaching levels approximately equal to or higher than the basal EL-4 T cell acetylation levels at the *GM-CSF* gene promoter (Figure 4.2; $P<0.05$, unpaired t-test).

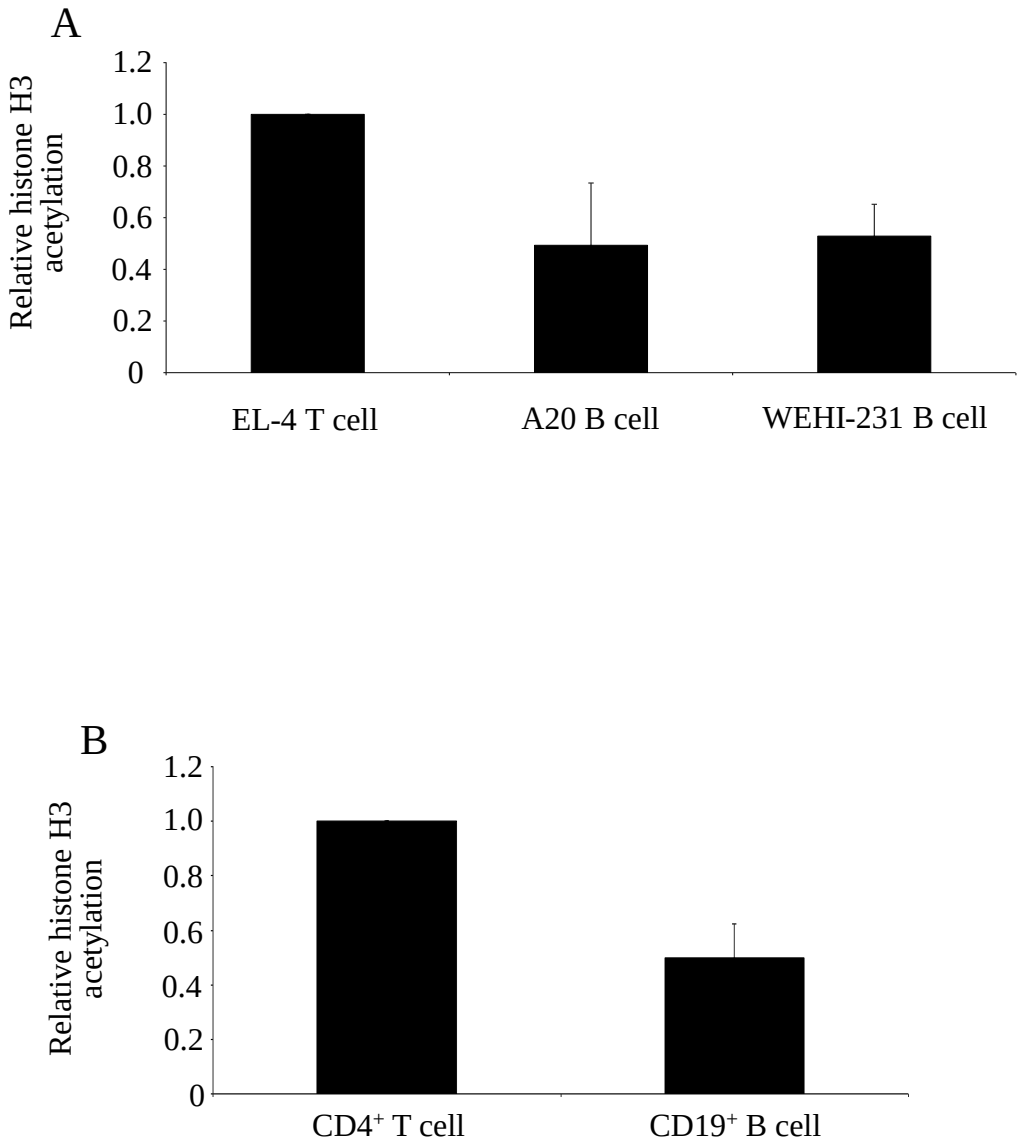

Figure 4.1: Histone H3 acetylation levels are higher at the *GM-CSF* promoter in T cells compared to B cells. A: Levels of acetylated histone H3 at the *GM-CSF* promoter were determined by qPCR analysis of ChIP in EL-4 T cells and A20 and WEHI-231 B cells. Acetylated H3 levels are shown relative to EL-4 T cells. The mean and standard error of three independent experiments are shown. B: Levels of acetylated histone H3 were determined at the *GMCSF* promoter in primary $CD4^+$ T cells and $CD19^+$ B cells as above. Levels are shown relative to $CD4^+$ T cells. The mean and standard error of two independent experiments are shown.

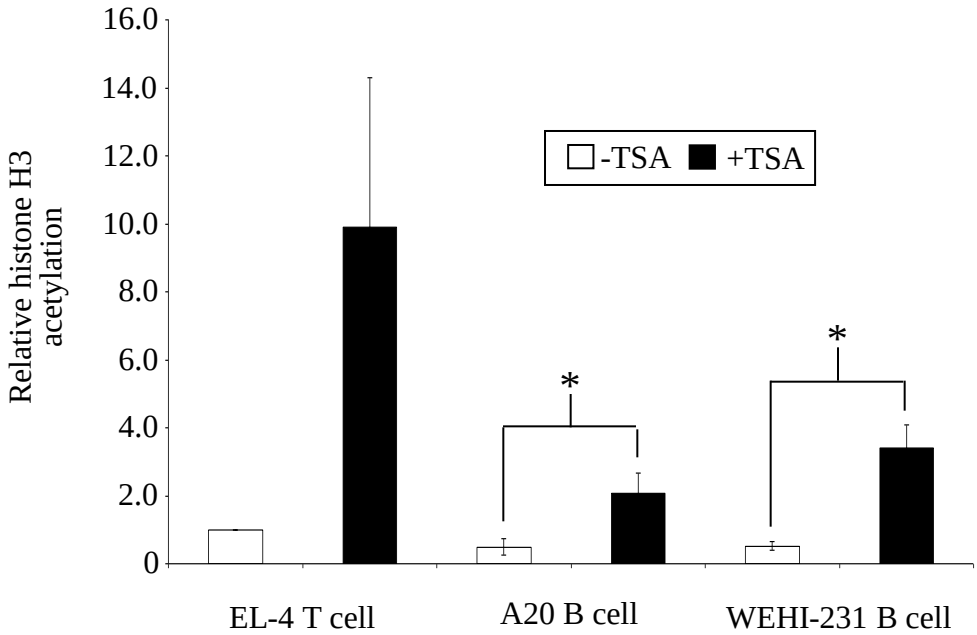

Figure 4.2: Trichostatin A treatment increases histone H3 acetylation levels at the *GM-CSF* promoter. EL-4 T cells, A20 B cells and WEHI-231 B cells were either left untreated or treated with TSA for 4 hours. Levels of acetylated histone H3 at the *GM-CSF* promoter were determined by ChIP-PCR. Levels are shown relative to EL-4 T cells. The mean and standard error of three independent experiments are shown. *P<0.05, unpaired t-test.

Since TSA was able to increase levels of promoter H3 acetylation in all three cell lines, next the effect of TSA treatment on *GM-CSF* gene expression was monitored. Cells were either left untreated or treated with TSA for 4 hours followed by a further 4 hour incubation with or without PI stimulation. RNA was harvested, and *GM-CSF* mRNA levels determined by RT-qPCR. In EL-4 T cells, as observed previously, PI treatment increased *GM-CSF* gene expression. While treatment with TSA alone did not affect *GM-CSF* gene expression, when EL-4 T cells were treated with TSA followed by PI stimulation, *GM-CSF* mRNA levels significantly increased to a level approximately 3.5 fold that seen with PI treatment alone (Figure 4.3a; $P<0.05$, unpaired t-test). In A20 B cells, treatment with PI or TSA alone had minimal effect on *GM-CSF* mRNA levels. However, following pretreatment with TSA an increase in *GM-CSF* mRNA levels was detected in response to PI stimulation (Figure 4.3b). Similarly, in WEHI-231 B cells, PI stimulation increased expression of *GM-CSF* mRNA in TSA pretreated cells. However, in this B cell line TSA treatment alone also resulted in a small increase in *GM-CSF* gene expression over basal levels (Figure 4.3c). To determine whether the observed effect of TSA on gene expression was a common response of all cytokines, the effect of TSA treatment on expression of the *IL-2* cytokine gene was also assessed in EL-4 T cells and WEHI-231 B cells. Stimulation with PI induced high levels of *IL-2* expression in EL-4 T cells, but not WEHI-231 B cells, as expected. However, pre-treatment with TSA did not affect *IL2* gene expression in either cell line (Figure 4.3d). This suggests that TSA treatment is having a specific effect on the *GM-CSF* gene in the T and B cell lines.

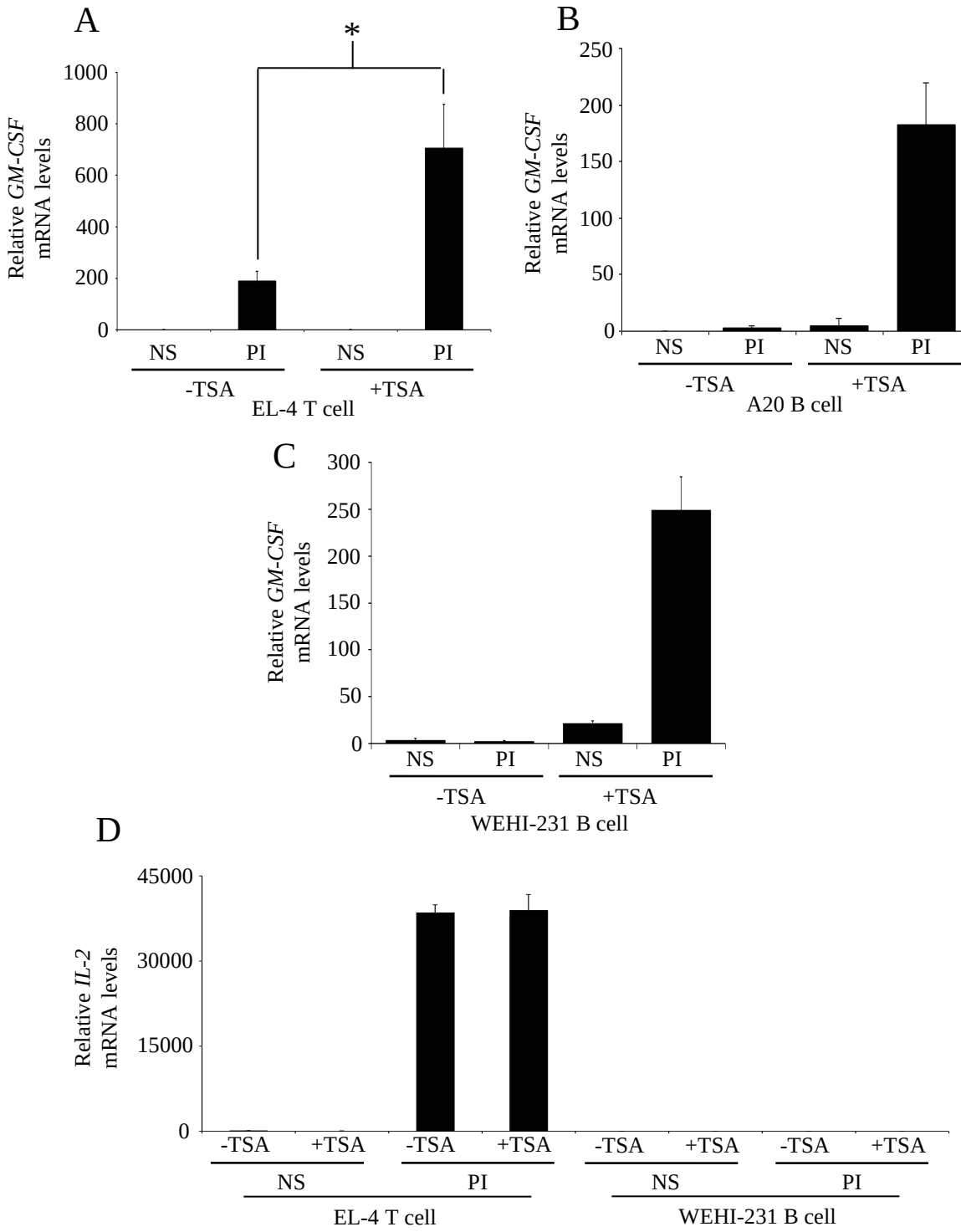

Figure 4.3: Trichostatin A treatment facilitates *GM-CSF* gene activation in B cell lines. A: EL-4 T cells were either pretreated with TSA for four hours (+TSA) or left untreated (-TSA), before being either stimulated with PMA and calcium ionophore (PI) for an additional four hours or left unstimulated (NS) as indicated. *GM-CSF* gene expression was determined by qPCR analysis. *P<0.05, unpaired t-test. *GM-CSF* mRNA levels were normalised to the *GAPDH* housekeeping gene. B and C: As for A, but using A20 B cells and WEHI-231 B cells respectively. D: *IL-2* gene expression was assessed using mRNA from the EL-4 T cell (A) and WEHI-231 B cell (C) samples. For all graphs, the mean and standard error of three independent experiments are shown.

Since TSA facilitates *GM-CSF* gene expression in response to PI stimulation in transformed B cell lines, the effect of TSA treatment in primary B cell lines was examined. Primary $CD4^+$ T cells and $CD19^+$ B cells were either left untreated or treated with TSA and PI (as in Figure 4.3), and *GM-CSF* mRNA expression analysed by qPCR (Figure 4.4). In the $CD4^+$ T cells, *GM-CSF* mRNA levels increased following PI stimulation, while treatment with TSA alone did not affect *GM-CSF* gene expression. Treatment with TSA followed by PI stimulation reduced *GM-CSF* gene expression when compared to PI stimulation alone. However, it is likely that this is a general effect on gene expression. In the $CD19^+$ B cells, *GM-CSF* gene expression was not induced in response to TSA, PI, or a combination of the two treatments, contrary to what was observed in transformed cell lines.

In Chapter 3, it was observed that DNA demethylation of the *GM-CSF* promoter following azacytidine treatment had a positive effect on *GM-CSF* gene expression in EL-4 T cells, but not A20 B cells. It was theorised that increased DNA methylation of the promoter acted to regulate *GM-CSF* gene expression at the transcriptional level. Since TSA treatment was shown to facilitate *GM-CSF* gene expression in A20 B cells, the effect of combining TSA and azacytidine treatment on *GM-CSF* gene expression was examined. EL-4 T cells and A20 B cells were either left untreated or pretreated with azacytidine for 24 hours, followed by a 24 hour withdrawal. The azacytidine treated and untreated cells were next either treated with TSA for 4 hours or left untreated, and finally stimulated with PI for four hours. *GM-CSF* mRNA

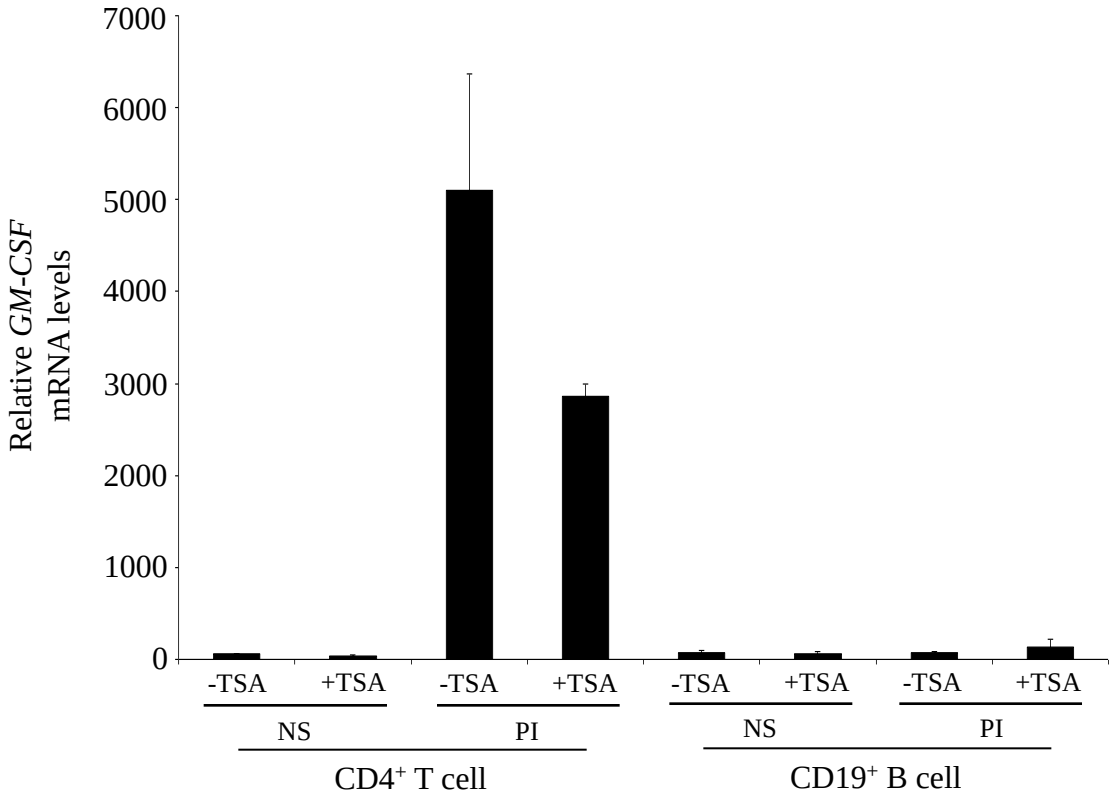

Figure 4.4: TSA does not facilitate *GM-CSF* gene activation in primary B cells. Primary $CD4^+$ T cells and $CD19^+$ B cells were either pretreated with TSA (+TSA) for 4 hours or left untreated (-TSA). Cells were then stimulated for 4h with PMA and calcium ionophore (PI) or left unstimulated (NS). *GM-CSF* gene expression was assessed by isolating mRNA and analysing by qPCR. *GM-CSF* mRNA levels were normalised to those of the *GAPDH* housekeeping gene. The mean and standard error of two independent experiments are shown.

levels were then analysed by RT-qPCR. In EL-4 T cells, as seen previously, treatment with TSA or azacytidine alone resulted in an increase in *GM-CSF* gene expression upon stimulation compared to that seen upon stimulation of untreated cells. Combined azacytidine and TSA treatment increased *GM-CSF* gene expression to a level greater than that of either TSA or azacytidine alone (Figure 4.5a). In A20 B cells, treatment with TSA facilitated *GM-CSF* gene activation upon stimulation with PI, as seen previously. Azacytidine pretreatment alone had little effect on *GM-CSF* gene expression following PI stimulation. However, treatment with both azacytidine and TSA resulted in a significant increase of *GM-CSF* mRNA levels in response to PI stimulation compared to TSA treatment alone, suggesting a synergistic effect between DNA demethylation and histone acetylation (Figure 4.5b; $P<0.05$, unpaired t-test), and supporting the notion that DNA methylation affects transcriptional activation.

As previously noted (Figure 4.1), histone H3 acetylation, a mark of transcriptionally permissive chromatin, is higher at the *GM-CSF* promoter in EL-4 T cells compared to A20 or WEHI-231 B cells. To determine if histone modifications associated with gene repression were reciprocally enriched at the *GM-CSF* promoter in B cells, the repressive histone modification trimethylation of lysine 27 on histone H3 (H3K27me3) was investigated. Levels of H3K27me3 were measured at four sites in EL-4 T cells and A20 B cells: the *GM-CSF* promoter, two regions located 1.1kb and 8kb upstream of the promoter, and the promoter of the rhodopsin gene, which is not expressed in these cell types, as a control (see Table 2.2 for primer information). In

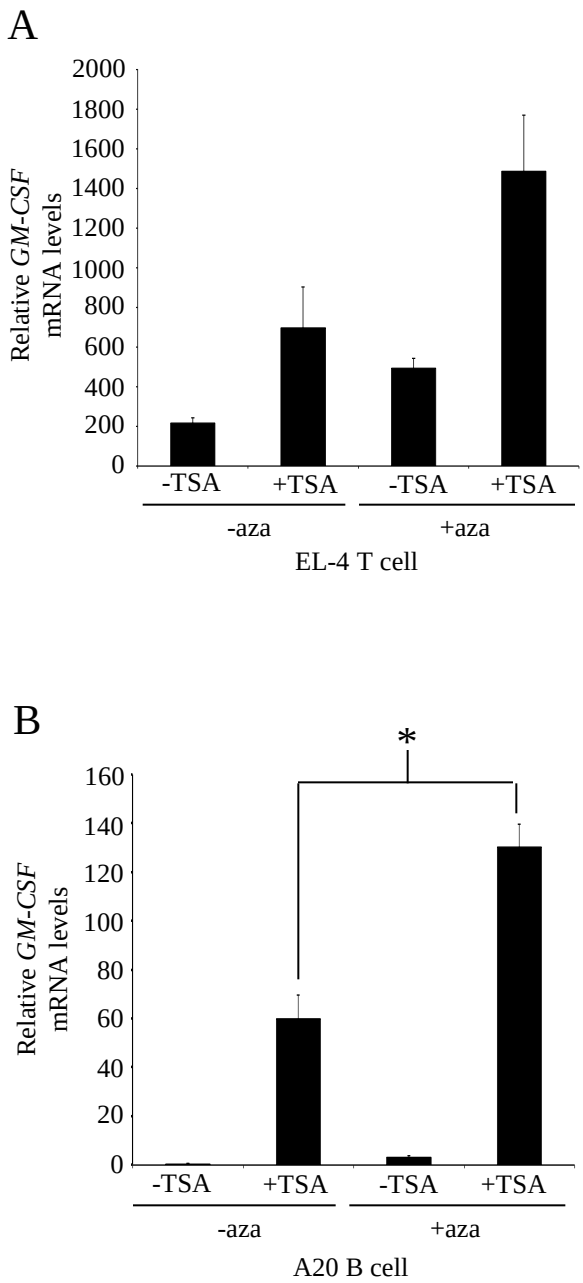

Figure 4.5: TSA and azacytidine synergistically activate *GM-CSF* gene expression. EL-4 T cells (A) and A20 B cells (B) were either left untreated (-aza) or pretreated with 5-azacytidine for 24 hours (+aza) followed by a 24 hour recovery period. Cells were then treated with TSA for 4 hours (+TSA) or left untreated (-TSA). Cells were then stimulated for 4 hours with PMA and calcium ionophore. *GM-CSF* gene expression was assessed by isolating mRNA and analysing by qPCR. *GM-CSF* mRNA levels were normalised to those of the *GAPDH* housekeeping gene. The mean and standard error of three independent experiments are shown. *P<0.05, unpaired t-test.

A20 B cells there was no difference in the level of H3K27me3 at all sites examined. At the *GM-CSF* promoter and the 1.1kb upstream site, levels of H3K27me3 were lower in EL-4 T cells when compared to A20 B cells, with a marginally significant difference observed at the promoter ($P<0.06$, unpaired t-test). At the 8kb upstream site and the inactive rhodopsin promoter, however, levels of trimethylation in the EL-4 T cells did not differ from those observed in the A20 B cells (Figure 4.6). This suggests that H3K27me3 levels are specifically lowered around the permissive *GM-CSF* promoter in EL-4 T cells. The results suggest that higher levels of H3K27me3 are present at the inactive genes: *GM-CSF* in the A20 B cells and rhodopsin in both T and B cells. However, at the *GM-CSF* gene in EL-4 T cells, which is able to express *GM-CSF* in response to stimulation, levels of H3K27me3 decrease towards the promoter. Therefore, enrichment of histone acetylation and decreased H3K27me3 levels appear to mark the *GM-CSF* promoter in expressing cell lines.

Activation of the *GM-CSF* gene in EL-4 T cells requires chromatin remodelling of a single nucleosome covering the *GM-CSF* promoter (Holloway *et al*, 2003; Brettingham-Moore *et al*, 2005; Chen *et al*, 2005), and it has been previously reported that the Brg1 protein, the core ATPase unit of the BAF chromatin remodelling complex, is enriched at the *GM-CSF* promoter in EL-4 T cells compared to A20 B cells (Brettingham-Moore *et al*, 2008). To determine whether this was also the case in primary cells, levels of Brg1 were examined at the promoter in primary $CD4^+$ T cells and $CD19^+$ B cells by ChIP. In $CD4^+$ T cells, the level of Brg1

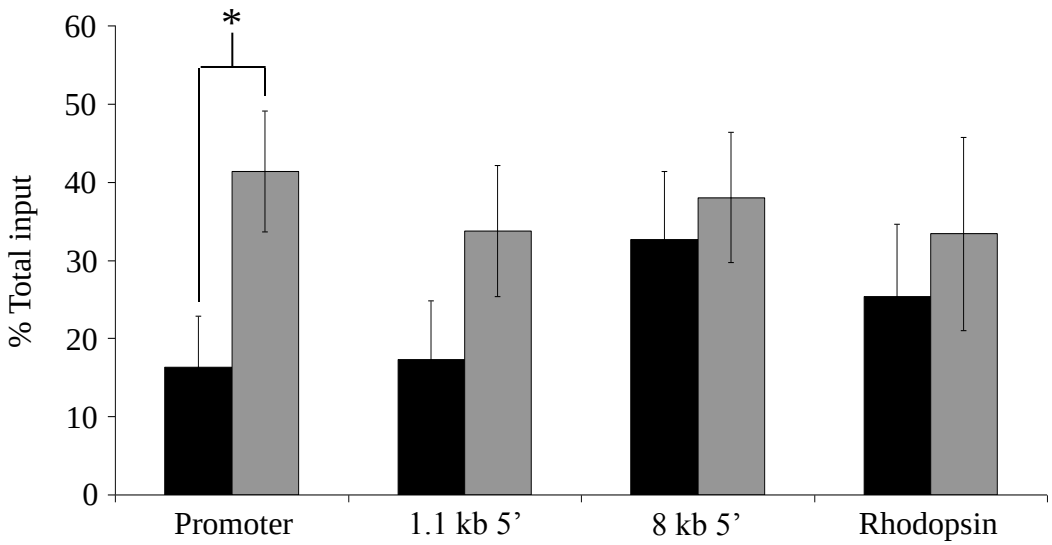

Figure 4.6: Histone H3 lysine 27 methylation at the *GM-CSF* promoter differs between T and B transformed cell lines. ChIP was performed on EL4 T cells (black) and A20 B cells (gray) using an anti-H3K27me3 antibody. Immunoprecipitated DNA was analysed by qPCR with primers against the *GM-CSF* promoter, two regions 1.1kb and 8kb upstream of the promoter, and the inactive rhodopsin promoter. Results are graphed as a percentage of total input DNA. The mean and standard error of three independent experiments is shown. Unpaired t-test used to analyse promoter samples (*P<0.06).

associated with the promoter was higher than that observed at the same site in $CD19^+$ B cells. At a site 1.1kb upstream, negligible levels of Brg1 were observed in both cell types (Figure 4.7), indicating that the enrichment of Brg1 is specific to the promoter region, and it is reduced in $CD19^+$ B cells compared to $CD4^+$ T cells.

The Mi-2β chromatin remodelling protein has been found to be enriched at the promoters of inducible genes in macrophages along with the SWI/SNF complex, with the two CRCs playing antagonistic roles. SWI/SNF was required to activate gene expression, while Mi-2β acted to repress it (Ramirez-Carrozzi *et al*, 2006). Therefore, since Brg1 was found to be enriched at the *GM-CSF* promoter, particularly in T cells, association of the Mi-2β chromatin remodelling protein at the *GM-CSF* promoter was also examined. Initially, nuclear proteins were isolated from EL-4 T cells and A20 B cells, separated by SDS-PAGE and analysed for Mi-2β by western blotting. Mi-2β was found to be present in the nuclei of both cell types at approximately equal levels, indicated by a band appearing in both samples at around the expected size of 210kD. The blot was also probed for Sp1 to demonstrate equal loading of both samples (Figure 4.8a). When levels of Mi-2β were examined by ChIP, Mi-2β was found to be enriched at the *GM-CSF* promoter compared to a 1.1kb upstream region in EL-4 T cells as well as A20 B cells. However, there was no difference in enrichment between the two cell lines (Figure 4.8b). In primary $CD4^+$ T cells and $CD19^+$ B cells, similar results were observed. Mi-2β was enriched at the *GM-CSF* promoter compared to the 1.1kb upstream region, but the enrichment at the

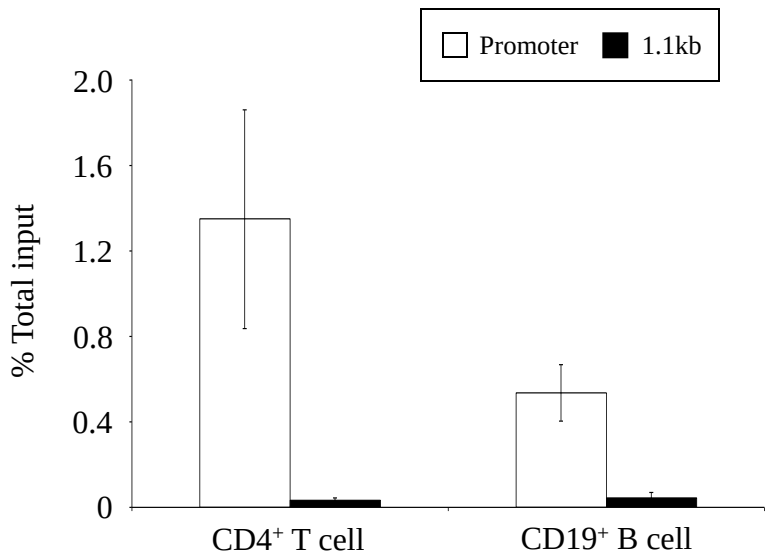

Figure 4.7: Brg1 is enriched at the *GM-CSF* promoter. $CD4^+$ T cells and $CD19^+$ B cells were subjected to ChIP using a Brg1 antibody. Immunoprecipitated DNA was analysed by qPCR using primers specific for the *GM-CSF* promoter or a region 1.1kb upstream. Results are graphed as a percentage of total input DNA. The mean and standard error of two independent experiments are shown.

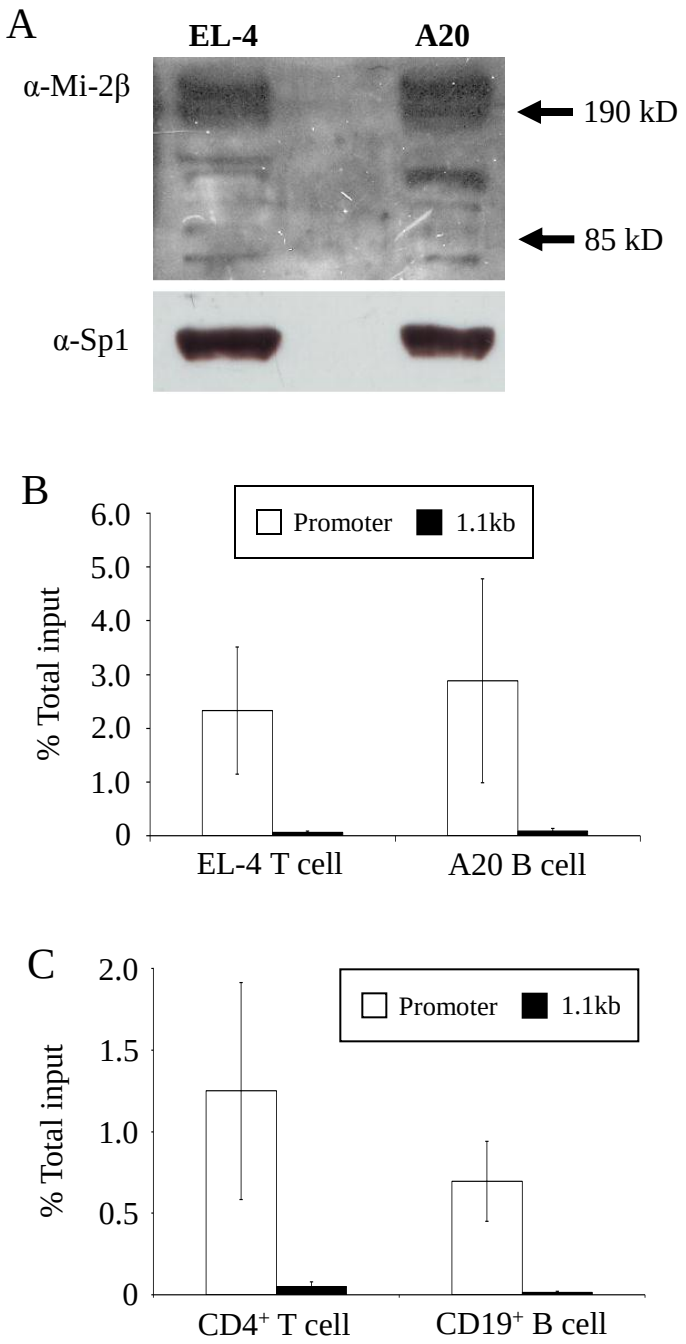

Figure 4.8: Mi-2β is enriched at the *GM-CSF* promoter in both T and B cells. A: Total nuclear protein (20µg) from EL-4 T cells and A20 B cells was separated by SDS-PAGE and analysed by Western blot using antibodies against Mi-2β and Sp1 as indicated. The expected size of Mi-2β is 210kD. Arrows indicate the location and size of bands of the protein ladder used. B: ChIP was performed on EL-4 T cells and A20 B cells using Mi-2β antibody. Immunoprecipitated DNA was analysed by qPCR using primers specific for the *GM-CSF* promoter or a region 1.1kb upstream. Results are graphed as a percentage of total input DNA. The mean and standard error of three independent experiments are shown. C: Primary $CD4^+$ T cells and $CD19^+$ B cells were analysed as for (B). The mean and standard error of two independent experiments are shown.

promoter did not differ between T and B cells (Figure 4.8c). Therefore, while both Brg1 and Mi-2β are enriched at the *GM-CSF* promoter in T and B cells, only Brg1 is differentially associated with the promoter between the two cell types, suggesting that this chromatin remodelling protein may contribute to the permissive chromatin state exhibited at the *GM-CSF* gene promoter in T cells.

4.3 - Discussion

These data illustrate that epigenetic differences exist at the *GM-CSF* gene between T cells and B cells. The *GM-CSF* promoter, in both transformed EL-4 T cells and primary $CD4^+$ T cells, possesses a higher level of acetylated histone H3 compared to the promoter in B cells (Figure 4.1). By using the histone deacetylase inhibitor Trichostatin A, hyperacetylation of the promoter could be achieved in EL-4 T cells and A20 and WEHI-231 B cells (Figure 4.2). This facilitated PI-stimulated expression of *GM-CSF* in the two B cell lines, which do not normally express *GM-CSF* in response to PI stimulation. In contrast, expression of the *IL-2* cytokine gene was not affected by TSA pretreatment (Figure 4.3). However, this is in contrast to a previous report (Rao *et al*, 2001) demonstrating increased inducibility of the *IL-2* gene following TSA treatment. There are a number of explanations for this discrepancy. In the data reported by Rao *et al* (2001), TSA was used at a higher concentration than in the experiments reported here (500ng/mL vs. 200ng/mL). Furthermore, following TSA treatment mRNA levels were measured after 1 hour of PI stimulation, while in the experiments presented here *IL-2* gene expression was measured after 4 hours of PI stimulation, which in Rao *et al* (2001) was demonstrated to be the time of peak *IL-2* mRNA levels following stimulation. This suggests that, in the case of the *IL-2* gene in EL-4 T cells, TSA pretreatment may act on the kinetics of gene activation, while not affecting the peak level of *IL-2* mRNA produced. Nevertheless, these data suggest that in the experiments reported here TSA treatment facilitates expression of the *GM-CSF* gene in response to PI stimulation, but does not cause indiscriminate de-repression of all cytokine genes.

While TSA was able to facilitate expression of *GM-CSF* in transformed B cell lines, the same was not true for primary B cells. When primary $CD19^+$ B cells were treated with TSA and stimulated under the same conditions as transformed cells lines, no increase in *GM-CSF* expression was observed. Furthermore, in primary $CD4^+$ T cells, TSA treatment prior to PI stimulation actually resulted in lower levels of *GM-CSF* expression compared to PI stimulation alone (Figure 4.4). Since T and B cells share similar patterns of histone H3 acetylation at the *GM-CSF* promoter between transformed and primary cell types, and increasing acetylation at the promoter via TSA treatment appears to activate *GM-CSF* expression in transformed B cell lines, this observation requires explanation. It has previously been reported that TSA treatment of primary murine $CD4^+$ T cells represses cytokine production and induces apoptosis (Moreira *et al*, 2003). Expression of the *IL-2* gene induced by anti-CD3/anti-CD28 treatment was found to be reduced in the presence of TSA. Treatment with TSA also generated reactive oxygen species and caused activation of the caspase-dependent cell death pathway. Such an effect may be responsible for the observed decrease in *GM-CSF* gene expression in TSA treated primary $CD4^+$ T cells and the lack of *GM-CSF* gene expression in TSA treated $CD19^+$ B cells. It is possible that transformed cell lines are more resistant to the apoptosis-inducing effects of TSA than primary cells, due to mutations incurred during the process of transformation. For example, it has been observed that EL-4 T cells are resistant to radiation-induced apoptosis compared to primary lymphocytes (Shankar *et al*, 2003).

In Chapter 3, it was found that demethylating the *GM-CSF* promoter with azacytidine resulted in increased *GM-CSF* gene expression in response to activating stimuli in EL-4 T cells but not A20 B cells. Since TSA overcame the block in *GM-CSF* gene expression in the transformed B cell lines, the effect of treatment with both azacytidine and TSA was examined. It was found that in both EL-4 T cells and A20 B cells, co-treatment of the cells with TSA and azacytidine caused a greater increase in *GM-CSF* gene expression in response to PI stimulation than the effect of either treatment alone (Figure 4.5). Particularly, in B cells, azacytidine alone did not affect *GM-CSF* gene expression, while co-treatment had a much greater effect than that of TSA alone. The synergistic effects of TSA and azacytidine have been noted in previous studies. For example, it has been shown that SK-N-SH cells, which normally do not express the citrate carrier gene (*CIC*), could be induced to express the gene upon treatment with TSA, azacytidine, or both, with the co-treatment having the greatest effect (Iacobazzi *et al*, 2008). It was hypothesised in Chapter 3 that DNA methylation of the *GM-CSF* promoter acted at the transcriptional level of gene expression, rather than the chromatin level. We have previously reported that TSA pretreatment relieves the block in chromatin remodelling of the *GM-CSF* promoter in A20 B cells (Brettingham-Moore *et al*, 2008), and the effect of azacytidine treatment on *GM-CSF* gene expression in A20 B cells is seen only in concert with TSA pretreatment, supporting the hypothesis. In this model, remodelling of the *GM-CSF* promoter chromatin (facilitated by TSA pretreatment in A20 B cells) is required before the methylation level (affected by azacytidine treatment) is able to exert an

effect on *GM-CSF* activation. This would explain the synergistic effects of these treatments: one affecting chromatin remodelling, the other gene transcription.

Apart from histone acetylation, differences were also observed between T and B cells in the distribution of the histone H3 lysine 27 trimethylation mark at the *GM-CSF* promoter. In EL-4 T cells and A20 B cells, H3K27me3 appears to be at similar levels between the cell types at regions other than the *GM-CSF* promoter; 8kb upstream of the promoter, and at the promoter of the inactive rhodopsin gene. Nearer to the transcriptional start site (1.1kb upstream) and at the promoter, H3K27me3 levels appear to be lowered in EL-4 T cells while remaining high in A20 B cells. This correlates with previous studies that have identified high levels of H3K27me3 at the promoters of repressed genes and low levels at the promoters of active genes. In high-resolution profiling of histone methylation patterns across the genome of human $CD4^+$ T cells, Barski *et al* (2007) found that inactive genes were characterized by high levels of H3K27me3 at the promoter and throughout the gene body. Active genes, on the other hand, showed reduced levels of H3K27me3.

Previous work investigating the *GM-CSF* gene found that in EL-4 T cells, the Brg1 chromatin remodelling complex is highly enriched at the *GM-CSF* promoter compared to A20 B cells (Brettingham-Moore *et al*, 2008). Here enrichment of Brg1 at the *GM-CSF* promoter in $CD4^+$ T cells compared to $CD19^+$ B cells was also demonstrated (Figure 4.7). Negligible enrichment was observed at the 1.1kb upstream region. This suggests that the role of Brg1 and the SWI/SNF chromatin

remodelling complex in regulating *GM-CSF* gene expression is conserved between primary and transformed cells.

Several studies have found that the SWI/SNF and NuRD (nucleosome remodelling and histone deacetylation) CRCs can be co-recruited to gene promoters, and play antagonistic roles in the regulation of gene expression. Ramirez-Carrozzi *et al* (2006) showed that Mi-2β (the core ATPase unit of the NuRD CRC) is co-recruited with SWI/SNF to the regulatory regions of several secondary response genes in macrophages. Promoter remodelling and activation of these genes upon stimulation was dependent on SWI/SNF, while depletion of Mi-2β resulted in an increase in gene activation. In plasmocytoma cells, a similar mechanism was implicated in the regulation of the *mb-1* gene. Knockdown of Brg1/Brm inhibited the response of the gene to activating stimuli, while knockdown of Mi-2β enhanced chromatin accessibility and gene activation (Gao *et al*, 2009). Since Brg1 is known to play a role in *GM-CSF* gene activation, ChIP was performed to determine the presence of Mi-2β at the *GM-CSF* promoter in T and B cells. However, while Mi-2β was found to be specifically enriched at the *GM-CSF* promoter compared to a 1.1kb upstream control region in both transformed and primary cells, there was no difference in Mi2β recruitment at the promoter between T and B cells (Figure 4.8). The fact that recruitment of Mi-2β is equivalent across cell types while Brg1 recruitment differs between T and B cells may suggest that Brg1 is more likely to be responsible for the differential expression seen between T and B cells. This is not to suggest, however, that Mi-2β does not play a role in *GM-CSF* gene regulation, as it is specifically

enriched at the *GM-CSF* promoter compared to a control region. In the Ramirez-Carrozzi *et al* (2006) study discussed above, depletion of Mi-2β by RNAi in macrophages resulted in increased expression of the genes studied upon stimulation, compared to a control. This suggests a role for Mi-2β in modulating the level of gene expression in response to a signal. It is possible that it has a similar role at the *GM-CSF* promoter.

The data presented in this chapter have begun to characterise the chromatin environment of the *GM-CSF* gene in immune cells, and it is clear that a number of differences in chromatin modification and chromatin remodelling complex recruitment at the *GM-CSF* promoter exist between T and B cells. One observation from this chapter is that the degree of *GM-CSF* promoter H3 acetylation and Brg1 recruitment to the promoter are correlated in both transformed and primary cells, with *GM-CSF* expressing T cells showing a higher level of both promoter acetylation and Brg1 recruitment. This suggests a possible mechanism for Brg1 recruitment. The Brg1 protein contains a bromodomain, which has been shown *in vitro* to bind to acetylated lysine residues (Hassan *et al*, 2002; Shen *et al*, 2007). An increase in promoter acetylation might therefore result in an increase in Brg1 binding. In EL-4 T cells, treatment with TSA has been shown to increase Brg1 recruitment to the GM-CSF promoter (Brettingham-Moore *et al*, 2008). For this thesis, the experiment was repeated using A20 B cells, and while the results were not statistically significant, a slight trend towards increased Brg1 recruitment at the promoter was observed in TSA

treated cells (data not shown). Future work could investigate this potential mechanism further, for example by means of a bromodomain deletion mutant.

Chapter Five - Transcriptional downregulation and resetting of chromatin at the *GM-CSF* promoter

5.1 - Introduction

While many studies have now been undertaken examining the role of chromatin remodelling and nucleosome displacement in gene activation (reviewed in Workman, 2006), less is known about how histones are redeposited onto DNA following removal of the activation signal, and the role this plays in transcriptional downregulation. Most of our knowledge of mechanisms of histone deposition has come from studies performed in yeast, primarily investigating the *PHO5* gene.

The *PHO5* gene has been widely used as a model system to investigate the role of chromatin in regulating inducible gene transcription, and has provided some of the earliest information on mechanisms of chromatin remodelling (see Chapter 1.3). Upon activation of the *PHO5* gene in response to low phosphate growth conditions, the promoter becomes accessible to nuclease attack across a four-nucleosome region, exposing a transcription factor binding site, the TATA box and the TSS (Svaren & Hörz, 1997). This increase in accessibility of the promoter is due to the complete loss of the promoter nucleosomes from the DNA (Boeger *et al*, 2003). Adkins *et al* (2004) observed that reassembly of chromatin onto the promoter correlates with gene repression. It was further demonstrated that this reassembly of nucleosomes is mediated by the histone chaperone protein Spt6 (suppressor of Ty 6). Furthermore, in the absence of Spt6 and nucleosome reassembly, *PHO5* transcription continued

despite the loss of the activating Pho4 and Pho2 transcription factors from the gene promoter. Further, it was reported that in the absence of Spt6 activity, the nucleosome-free transcriptionally active state of *PHO5* is maintained even following DNA replication (Ohsawa *et al*, 2009). The *PHO8* gene and the glucose-regulated genes alcohol dehydrogenase 2 (*ADH2*), accumulation of dyads proteins 2 (*ADY2*), and invertase (*SUC2*) were also found to undergo nucleosome loss upon activation and Spt6-dependent nucleosome reassembly during transcriptional downregulation (Adkins *et al*, 2006), suggesting a common mechanism for histone reassembly and gene repression, at least in yeast. Schermer *et al* (2005) showed that the histones redeposited onto the *PHO5* promoter originate from the soluble pool of non-assembled histones (in *trans*), as opposed to histones already assembled into the non-soluble chromatin fraction (in *cis*). The chaperones Asf1 and histone regulation 1 (Hir1) were also implicated in promoter reassembly, as well as the SWI/SNF complex.

Reassembly of chromatin and the role it plays in transcriptional downregulation has also been examined at other genes. Jensen *et al* (2008) demonstrated a role for Spt6 and Spt16 (a subunit of the FACT complex) in resetting chromatin structure and downregulating expression of the yeast gene heat shock protein 104 (*HSP104*). An Spt6 mutant is incapable of restoring the chromatin structure of the gene, but gene transcription is downregulated even in the absence of chromatin resetting. When Spt16 activity was perturbed, however, both chromatin resetting and transcriptional downregulation did not occur. Chromatin reassembly and the Spt6 chaperone have

also been shown to play a role in the transcriptional downregulation of *HIV-1* transcription in a yeast-based system (Vanti *et al*, 2009).

While the studies outlined above have provided some insight into the role of histone deposition in transcriptional downregulation of gene expression, little is known about such mechanisms in mammalian cells. Chen *et al* (2005) demonstrated that histones are lost from the *IL-2* promoter upon murine T cell activation and histones reassociate with the promoter following stimulus withdrawal. Similarly, previous work on the *GM-CSF* gene has shown that activating stimuli cause an increase in both promoter accessibility and *GM-CSF* mRNA levels. Following stimulus withdrawal, *GM-CSF* promoter accessibility decreases and mRNA levels return to baseline (Brettingham-Moore *et al*, 2005). This implies that reassembly of chromatin at the *GM-CSF* promoter may be important in downregulating transcription of the *GM-CSF* gene. Given the dearth of information on the role of promoter chromatin reassembly in transcriptional downregulation of mammalian genes, further investigation of the mechanisms of chromatin reassembly and its affect on gene transcription are warranted. Additionally, since it was demonstrated in Chapter 4 that a permissive chromatin state could be generated in A20 B cells by TSA treatment, the fate of this induced permissive state following transcriptional downregulation and chromatin resetting may provide additional insight into the mechanisms involved.

The aim of this chapter was therefore to examine resetting of the chromatin environment of the *GM-CSF* promoter following stimulus withdrawal.

5.2 - Results

To examine chromatin dynamics at the *GM-CSF* promoter following gene activation and subsequent transcriptional downregulation, a timecourse was devised to allow both of these events to be examined (Figure 5.1). These events were monitored in both EL-4 T cells and A20 B cells. Cells were treated with TSA for 4 hours, followed by 4 hours of PI stimulation. At this point, the stimulus was removed from the cells and the cells were grown for a further 20 hours, before being restimulated with PI for 4 hours. Samples for analysis were taken before treatment (baseline or BL timepoint, see Figure 5.1), following TSA and PI stimulation (T/P timepoint), following the 20 hour stimulus withdrawal (stimulus removed or SR timepoint) and following the second PI stimulation (restimulated or RS timepoint).

Initially, the expression of *GM-CSF* mRNA at these timepoints was examined in EL-4 T cells and A20 B cells (Figure 5.2). At the baseline timepoint, neither cell type expressed *GM-CSF* mRNA, as expected. Following TSA and PI treatment, an increase in the expression of *GM-CSF* mRNA was observed in both cell types, with expression returning to basal levels following 20 hours of stimulus removal. However, the two cell types exhibited different responses to a second PI stimulation. In EL-4 T cells, expression of the *GM-CSF* gene following restimulation increased to greater levels than those observed following the initial stimulation (Figure 5.2a). Conversely, in A20 B cells, no increase in *GM-CSF* gene expression was observed

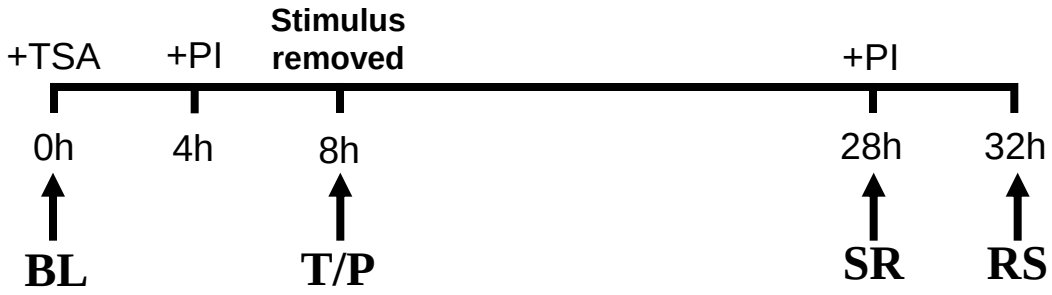

Figure 5.1: Timecourse for examining chromatin resetting at the *GM-CSF* promoter. A timecourse of cell treatment is shown, with the treatment indicated above and the timepoints (in hours) and sampling times (black arrows) indicated below. At 0 hours, no treatment has been applied and thus the cells are at **baseline** (**BL**). At 8 hours, the cells have been treated with **TSA and PI** (**T/P**). At 28 hours, the cells have had the **stimulus removed** (**SR**) for 20 hours, and at 32 hours the cells have been **restimulated** (**RS**) with PI for 4 hours.

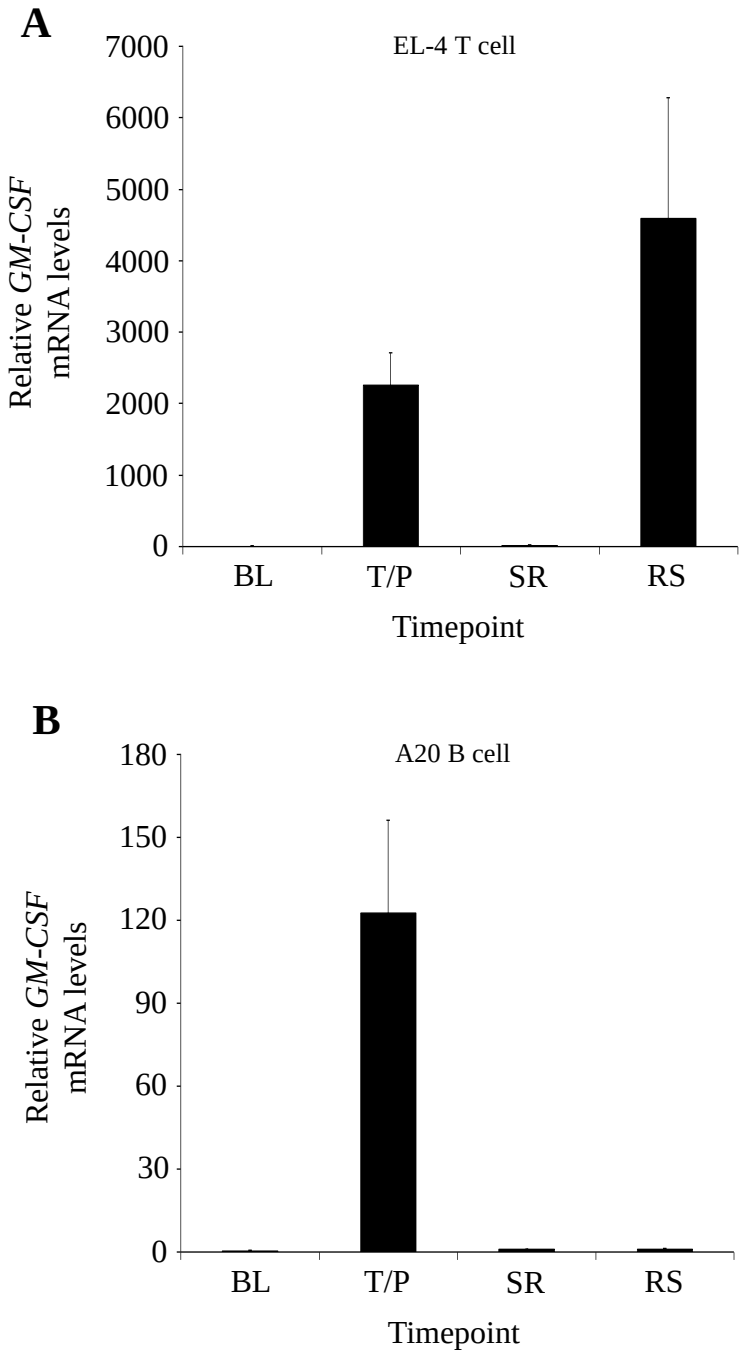

Figure 5.2: The *GM-CSF* gene responds differently to restimulation in EL-4 T cells and A20 B cells. EL-4 T cells (A) and A20 B cells (B) were treated according to the timecourse shown in Fig. 5.1. At each time point, mRNA was extracted, cDNA prepared, and *GM-CSF* gene expression analysed by qPCR. *GM-CSF* gene expression is shown normalised to *GAPDH*. The mean and standard error of four independent experiments are shown. BL, base line; T/P, TSA/PI treatment, SR, stimulus withdrawal; RS, restimulation.

following restimulation (Fig 5.2b). This suggests that while in A20 B cells a permissive chromatin environment can be generated at the *GM-CSF* promoter by pre-treatment with TSA, this permissive state is not maintained following stimulus removal, and the chromatin is reset to its basal repressive state in A20 B cells following stimulus withdrawal.

To investigate this, chromatin dynamics at the *GM-CSF* promoter were examined during the same time course using the CHART-PCR MNase accessibility assay, as described in Rao *et al* (2001) (Figure 5.3). The percentage accessibility at each timepoint was calculated, and the results graphed as the change in accessibility from the baseline timepoint. In both EL-4 T cells and A20 B cells, an increase in MNase accessibility at the *GM-CSF* promoter was observed following stimulation with TSA and PI. This increase in accessibility is indicative of chromatin remodelling at the promoter. Following removal of the stimulus for 20 hours, a decrease in promoter chromatin accessibility was observed in both cell types. Upon restimulation, the level of accessibility at the promoter in the EL-4 T cells increased, while in A20 B cells, no change in accessibility was detected following restimulation. This is in keeping with the differential pattern of *GM-CSF* gene expression observed between the cell types following restimulation, and suggests that the basal chromatin environment of the *GM-CSF* promoter is reset following stimulus removal. In the case of A20 B cells this involves a return to the repressive chromatin state.

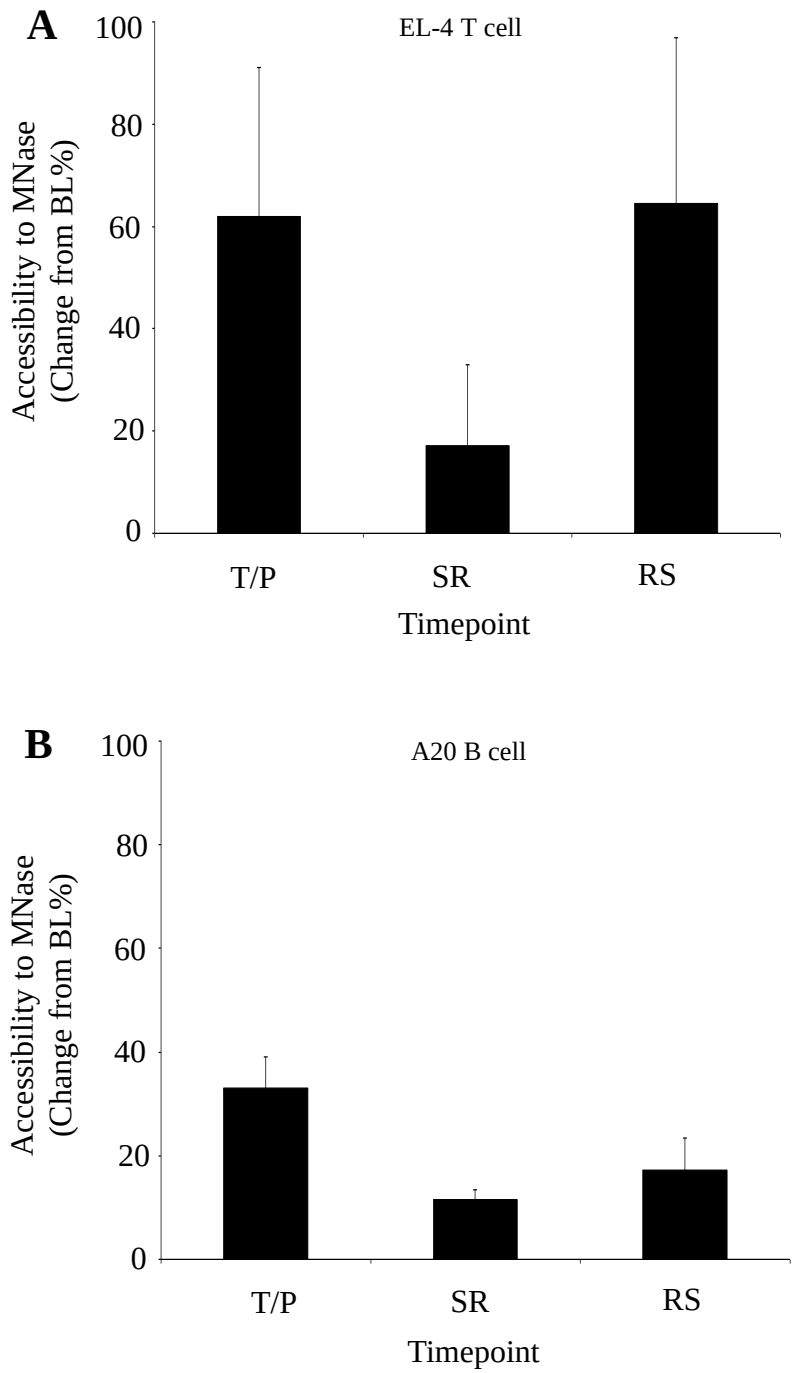

Figure 5.3: Chromatin remodelling at the *GM-CSF* promoter in response to restimulation occurs in EL-4 T cells but not A20 B cells. EL-4 T cells (A) and A20 B cells (B) were treated as indicated in Fig. 5.1. Nuclei were extracted at each time point and chromatin digested with MNase for CHART-PCR analysis of the *GM-CSF* promoter region. Percentage accessibility at each timepoint was calculated, and the results graphed as the change in accessibility from the baseline timepoint. The mean and standard error of three independent experiments are shown. BL, base line; T/P, TSA/PI treatment; SR, stimulus withdrawal; RS, restimulation.

To determine if the increase in chromatin accessibility observed in A20 B cells in response to TSA/PI treatment was associated with promoter histone loss, levels of histone H3 at the *GM-CSF* promoter were measured across the timecourse in A20 B cells using chromatin immunoprecipitation (Figure 5.4). Following treatment with TSA and stimulation with PI, there was a significant decrease in the relative level of H3 at the *GM-CSF* promoter, to approximately half the baseline level (one sample t-test, $p<0.05$). This correlates with the observed increase in promoter accessibility at the same timepoint (Figure 5.3). Following stimulus withdrawal, levels of H3 began to return towards those observed at baseline. Upon a second PI stimulation (RS), H3 levels did not change at the promoter, implying that chromatin remodelling does not occur upon restimulation. To determine if these changes were localised to the *GM-CSF* promoter, H3 levels were examined at a region 1.1kb upstream of the promoter, as a control (see Table 2.2 for primers used). At this upstream region, levels of H3 remained relatively stable across the timecourse, indicating that no or minimal disturbance of the chromatin is taking place. Interestingly, basal H3 occupancy at the upstream region is approximately 60% that of basal promoter H3 occupancy, suggesting a denser chromatin structure exists at the *GM-CSF* promoter than at the 1.1kb upstream region in A20 B cells.

The dynamics of histone modifications were also examined across the timecourse. Global histone H3 acetylation levels were assessed by isolation of nuclear protein from A20 B cells and analysis by western blotting (Figure 5.5). Global AcH3 levels increased as expected following TSA treatment (T/P) compared to basal levels.

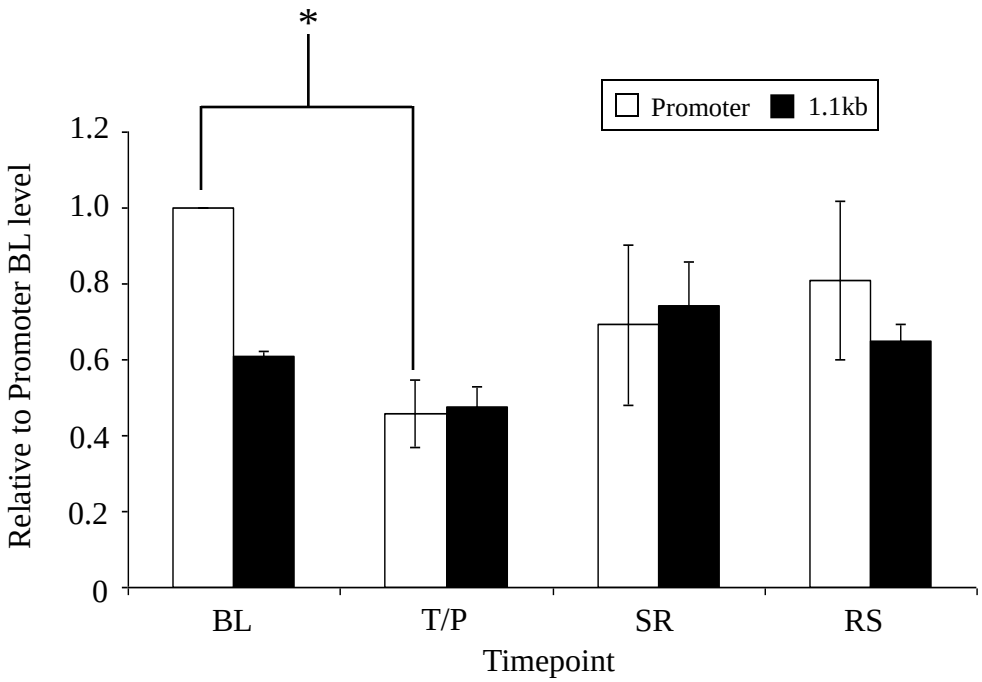

Figure 5.4: Histone H3 dynamics at the *GM-CSF* promoter in A20 B cells. A20 B cells were treated as in Fig. 5.1 and ChIP performed at each time point using an antibody against histone H3. DNA was analysed by qPCR using primers against the *GM-CSF* promoter region and a region 1.1kb upstream. The mean and standard error of three independent experiments are shown. *P<0.05, one sample t-test. BL, base line; T/P, TSA/PI treatment; SR, stimulus withdrawal; RS, restimulation.

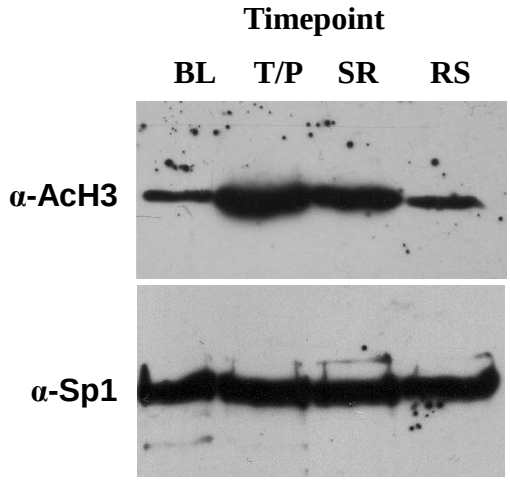

Figure 5.5: Global H3 acetylation patterns in A20 B cells. Nuclear proteins were extracted from A20 B cells at each point in the time course described in Fig. 5.1, separated by SDS-PAGE and analysed for acetylated histone H3 content by Western blot. Sp1 levels were also analysed as a loading control. BL, base line; T/P, TSA/PI treatment; SR, stimulus withdrawal; RS, restimulation.

Following stimulus removal for 20 hours (SR), AcH3 levels were still elevated above baseline. However following restimulation (RS) with PI for 4 hours (i.e. 24 hours post stimulus withdrawal), AcH3 levels had returned to approximately the same level seen at baseline. Levels of the Sp1 transcription factor, as examined by western blotting, were similar at all timepoints, indicating equal loading of protein samples.

Levels of acetylated histone H3 at the *GM-CSF* promoter were also assessed using ChIP (Figure 5.6). At baseline, AcH3 levels at the promoter were elevated over those observed at the 1.1kb upstream region, suggesting localised enrichment of AcH3 at the promoter, although this may at least partially reflect the higher histone density at the promoter. Upon TSA/PI treatment, levels of AcH3 at the promoter decreased significantly (one sample t-test, $P<0.05$) to less than half the basal level, presumably due to loss of histones from this region, as observed previously (Figure 5.4). Conversely, AcH3 levels at the 1.1kb upstream region were observed to increase, as might be expected following TSA treatment. Following 20 hours of stimulus withdrawal, the AcH3 level at the promoter was elevated compared to both of the previous timepoints. This suggests the reassembly of hyperacetylated histones at the promoter. In comparison, at the 1.1kb upstream region, levels had decreased slightly. Following restimulation (i.e. 24 hours post-stimulus withdrawal), promoter AcH3 levels decreased to a level comparable to that observed at baseline, while there was minimal change at the 1.1kb upstream region. Put together, this data suggests that treatment with TSA generates a transcriptionally permissive chromatin environment at the *GM-CSF* promoter, allowing loss of histones in response to an immune signal.

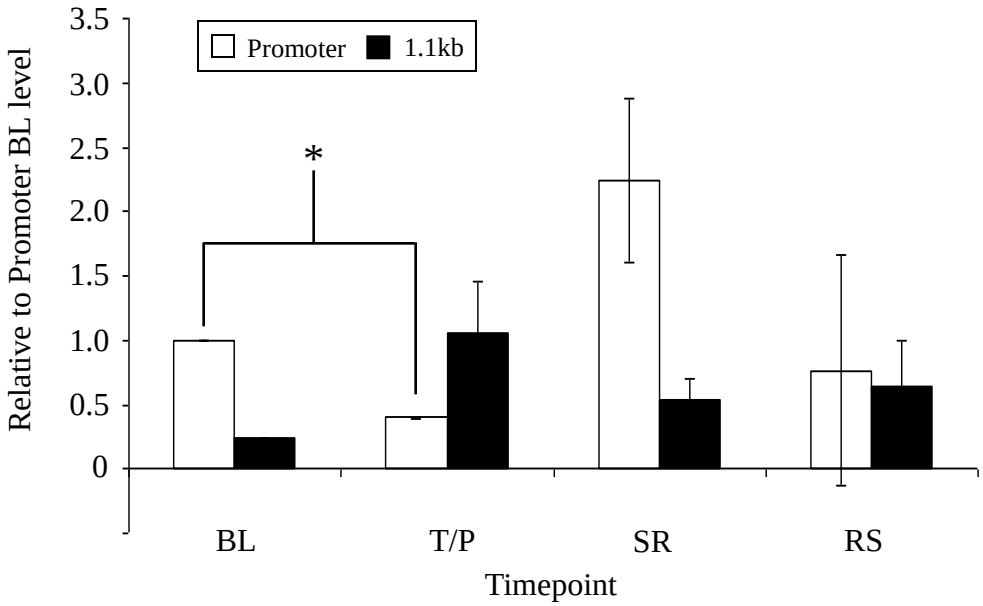

Figure 5.6: Acetylated histone H3 dynamics at the *GM-CSF* promoter in A20 B cells. Levels of AcH3 at the *GM-CSF* promoter and 1.1kb upstream region (used as a control) during the timecourse described in Fig. 5.1 were assessed by chromatin immunoprecipitation and qPCR. The mean and standard error of two independent experiments are shown. *P<0.05, one sample t-test. BL, base line; T/P, TSA/PI treatment; SR, stimulus withdrawal; RS, restimulation.

Following stimulus removal, hyperacetylated histones are reassembled at the promoter. Upon stimulation, a decrease in histone acetylation is observed, reflecting the decrease in global acetylation at this time, but chromatin remodelling at the promoter does not occur.

Inhibition of protein synthesis by cycloheximide (CHX) treatment has previously been shown to prevent *GM-CSF* gene activation by inhibiting chromatin remodelling events at the gene promoter (Brettingham-Moore *et al*, 2005). This is due to a requirement for NF-κB proteins for chromatin remodelling. To determine whether *GM-CSF* gene activation in A20 B cells following generation of a permissive chromatin environment by TSA pre-treatment is similarly protein synthesis dependent, the effect of CHX on TSA-induced *GM-CSF* gene expression in A20 B cells was examined. A20 B cells were either left untreated or pretreated with CHX for 30 minutes, followed by incubation with or without TSA for 4 hours. Cells were then stimulated with PI for 4 hours or left unstimulated. RNA was then isolated and cDNA prepared for analysis by qPCR. Increased *GM-CSF* mRNA levels were observed as previously following PI stimulation of TSA treated cells, but this effect was inhibited by CHX pretreatment (Figure 5.7)

To determine whether transcriptional downregulation of the *GM-CSF* gene following stimulus withdrawal was similarly protein synthesis dependent, A20 B cells were treated as before (see Figure 5.1), but following stimulus removal, cells were treated with or without CHX and the effects on *GM-CSF* gene expression examined. As

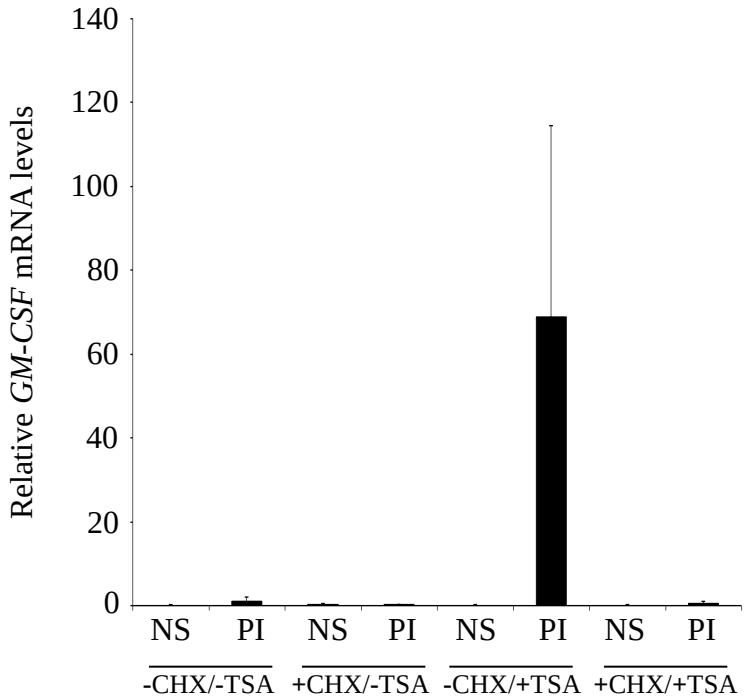

Figure 5.7: Inhibition of protein synthesis prevents *GM-CSF* gene activation in response to TSA treatment in A20 B cells. A20 B cells were treated with or without cycloheximide (CHX) for 30 minutes, followed by treatment with or without TSA for 4 hours, as indicated. Cells were then stimulated with PI for 4 hours or left non-stimulated (NS). RNA was extracted and expression of *GM-CSF* mRNA determined by qPCR. Data is graphed normalised to *GAPDH* and relative to the untreated sample. The mean and standard error of two independent experiments are shown.

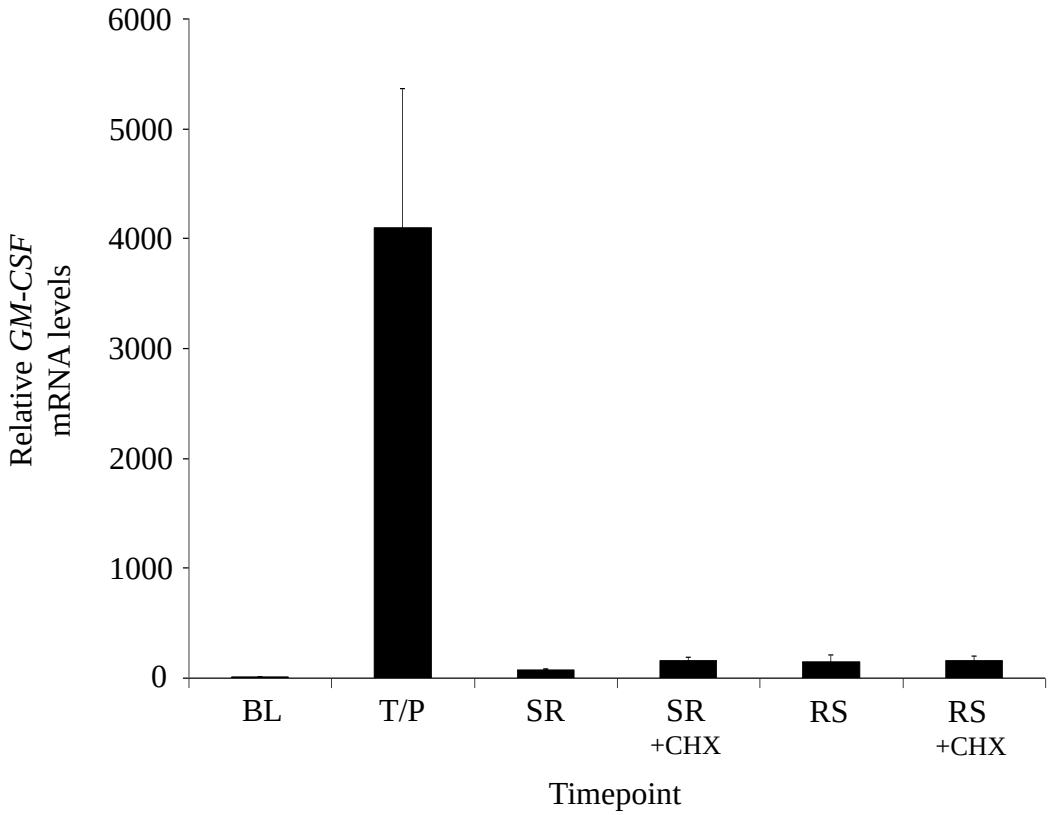

Figure 5.8: Cycloheximide treatment following stimulus withdrawal does not prolong *GM-CSF* gene expression in A20 B cells. A20 B cells were treated as indicated in Fig. 5.1. At the T/P timepoint, half of the cells were treated with CHX for the remainder of the experiment, with the other half treated normally, as indicated. RNA was extracted and expression of *GM-CSF* mRNA determined by qPCR. *GM-CSF* expression is shown normalised to *GAPDH*. The mean and standard error of three independent experiments are shown. BL, base line; T/P, TSA/PI treatment, SR, stimulus withdrawal; RS, restimulation.

seen previously, when the stimulus is removed *GM-CSF* mRNA levels return to basal levels, and CHX treatment had no effect on this (Figure 5.8). Restimulation with PI had no effect on *GM-CSF* mRNA expression either in the presence or absence of CHX (Figure 5.8). Therefore, while activation of the transcriptionally competent *GM-CSF* promoter in A20 B cells requires protein synthesis, repression of gene expression following stimulus removal does not.

5.3 - Discussion

The data presented here demonstrate that while a transcriptionally permissive state at the *GM-CSF* promoter can be generated in A20 B cells by TSA treatment, it is not maintained following withdrawal of TSA and resetting of the chromatin structure. Both T and B cells express the *GM-CSF* gene in response to TSA treatment and PI stimulation; however, following withdrawal of the stimulus and transcriptional downregulation, A20 B cells do not express *GM-CSF* in response to a second stimulation, while EL-4 T cells do (Figure 5.2). This pattern of *GM-CSF* gene expression was mirrored by changes in accessibility at the *GM-CSF* promoter, indicating that chromatin resetting takes place (Figure 5.3). The differential response of the *GM-CSF* gene to restimulation indicates that a form of epigenetic memory may be present at the *GM-CSF* promoter.

Specifically, it appears that in A20 B cells, the promoter chromatin is reset to a state that does not contain the induced histone modifications that facilitated *GM-CSF* gene expression initially. Following TSA treatment and stimulation, a significant decrease in the level of histone H3 at the *GM-CSF* promoter was observed in A20 B cells (Figure 5.4). This occurred concurrently with an increase in chromatin accessibility, suggesting that the increased accessibility is due to nucleosome loss at the promoter. The observed decrease in histones does not occur at a region 1.1kb upstream of the *GM-CSF* promoter, implying that this chromatin remodelling is specific to the promoter region. Specific nucleosomal remodelling at the *GM-CSF* promoter in response to stimulation has previously been observed in EL-4 T cells (Holloway *et*

al, 2003). Following withdrawal of TSA and the PI stimulus, histone H3 levels at the *GM-CSF* promoter were observed to increase. This occurred concomitant with a decrease in both chromatin accessibility and *GM-CSF* gene expression, suggesting a link between nucleosomal reassembly at the promoter and transcriptional downregulation. This pattern of specific nucleosome remodelling at promoter regions upon stimulation followed by nucleosome reassembly during transcriptional downregulation has previously been reported for the yeast genes *PHO5*, *PHO8*, *ADH2*, *ADY2* and *SUC2* (Adkins *et al*, 2004; Adkins *et al*, 2006), suggesting that it may be a common feature of inducible gene regulation.

As reported in Chapter 4, treatment of A20 B cells with TSA causes an approximately fourfold increase in levels of acetylated histone H3 at the *GM-CSF* promoter (Figure 4.2) and facilitates *GM-CSF* gene expression in response to PI stimulation (Figure 4.3b). Following TSA treatment and PI stimulation of A20 B cells, a significant decrease in AcH3 levels at the *GM-CSF* promoter was observed (Figure 5.6). The magnitude of the decrease was similar to that observed at the same region for histone H3 (Figure 5.4), suggesting that this decrease is due to loss of histone H3 overall, rather than active histone deacetylation. At the 1.1kb upstream region, however, an increase in AcH3 levels from baseline was observed following TSA/PI treatment. Since histone H3 levels do not decrease at this region, and global levels of AcH3 were higher at this timepoint compared to baseline (Figure 5.5), this increase is most likely due to the global effects of TSA on histone acetylation levels. Following the withdrawal of TSA and PI, AcH3 levels at the *GM-CSF* promoter

increased, while those at the 1.1kb upstream region decreased. Since global levels of AcH3 at this timepoint are still elevated compared to baseline, the increase in AcH3 at the promoter may be due to reassembly of these highly acetylated histones onto the promoter. In the case of the yeast *PHO5* gene, the histones assembled onto the gene promoter during transcriptional downregulation have been shown to originate in *trans* from the soluble pool of non-assembled histones, rather than being transferred in *cis* from nucleosomes already assembled on another region of DNA (Schermer *et al*, 2005). It has previously been reported that acetylation of newly synthesised histones takes place in the cytoplasm prior to nucleosomal assembly (Sobel *et al*, 1995) with deacetylation occurring after assembly of the histones into nucleosomes. It is therefore possible that the histones assembled onto the *GM-CSF* promoter during transcriptional downregulation originate in *trans* from the acetylated pool of soluble histones, leading to an apparent increase in histone acetylation at the *GM-CSF* promoter at this timepoint.

Upon restimulation, A20 B cells do not express the *GM-CSF* gene. Histone H3 levels at the *GM-CSF* promoter do not change compared to the earlier timepoint, and there is no observed increase in chromatin accessibility, while histone H3 acetylation at the promoter appears to decrease. The decrease in acetylation with no concurrent decrease in histone H3 levels suggests that this is not due to histone loss. It may instead be due to increased HDAC activity at the promoter as the effect of the previous TSA treatment diminishes, or exchange of acetylated promoter histones with non-acetylated histones from the soluble pool. The global level of histone H3

acetylation at this timepoint is lower than at the earlier timepoint, and therefore the histones in the soluble pool may be less highly acetylated than at earlier timepoints. In support of this, histone H3 exchange at promoters occurs during DNA replication, and has also been shown to take place independent of replication at the promoters of both active and inactive genes (Rufiange *et al*, 2007).

The fact that the A20 B cells do not express the *GM-CSF* gene following restimulation implies that the promoter chromatin is reset to the basal, unresponsive, state that existed prior to TSA treatment. The effect of TSA treatment is to increase histone acetylation due to inhibition of HDAC activity, as seen in Figure 4.2. Following stimulus removal, the promoter chromatin in A20 B cells appears to have higher levels of H3 acetylation compared to those seen at the baseline timepoint. This would suggest that the promoter chromatin is hyperacetylated and thus the gene should be responsive to PI stimulation, yet no *GM-CSF* gene expression was detected upon restimulation. There may be several explanations for this. Firstly, as shown in Figure 4.2, TSA treatment caused a roughly 4-fold change in AcH3 levels at the *GM-CSF* promoter in A20 B cells. However, when comparing AcH3 levels at the A20 B cell *GM-CSF* promoter between the baseline and stimulus removed timepoints in Figure 5.6, the change appears closer to 2-fold. Therefore, while there is an increase in promoter histone acetylation between the baseline and stimulus removal timepoints, it is of smaller magnitude than that induced by TSA treatment in Figure 4.2. Thus, the apparent increase seen following stimulus removal may not be sufficient to enable chromatin remodelling and gene transcription. Also, there may

be other factors recruited to the *GM-CSF* promoter as a consequence of TSA treatment that are displaced upon gene activation, and do not reassociate with the gene promoter when the chromatin is reassembled. Additionally, other repressive marks not affected by TSA treatment, such as DNA and histone methylation, may remain in the vicinity of the promoter and recruit repressive factors to the *GM-CSF* promoter following chromatin resetting.

Therefore, it is clear that in the case of the *GM-CSF* promoter in A20 B cells, while a transcriptionally permissive chromatin state can be induced by TSA treatment, it is not maintained following resetting of the chromatin. The mechanism of chromatin resetting is therefore of interest. We have previously observed that in EL-4 T cells, blocking *de novo* protein synthesis with CHX treatment immediately upon stimulus withdrawal has the effect of maintaining an open chromatin structure at the *GM-CSF* promoter and increased levels of *GM-CSF* transcription even after 20 hours of stimulus withdrawal (Upcher, Honours thesis 2007, unpublished). In A20 B cells, treating with CHX after withdrawing the stimulus did not cause prolonged expression of *GM-CSF*, even after a second stimulation. Therefore, resetting of the *GM-CSF* promoter chromatin in A20 B cells is likely not dependent on new protein synthesis.

While cycloheximide has been found to cause cell cycle arrest in primary cell lines, transformed cell lines have been shown to progress through the cell cycle in the presence of cycloheximide (Medrano and Pardee, 1980). As the A20 B cell line is transformed, progression through the cell cycle during the 20 hour stimulus

withdrawal might be responsible for the resetting of the *GM-CSF* promoter. To determine this, further work would need to be done to more accurately examine the effects of the cell cycle and the exact mechanisms of promoter resetting in both T and B cells. Using cell cycle blocking agents and flow cytometry analysis, populations of cells can be synchronised to the same stage in the cell cycle, allowing chromatin modifications and structure and gene expression to be mapped at precise points during DNA replication and cell division. Other proteins that may be involved in the mechanism of histone redeposition could also be examined. The studies referred to in the introduction of this chapter implicated the chaperone protein Spt6 in chromatin resetting. While these studies were performed in yeast, a murine homolog of Spt6 is known to exist: suppressor of Ty 6 homolog (Supt6h) (Chiang *et al*, 1996). However, a review of the literature reveals that this protein has only been studied in mouse models in relation to its role in RNA splicing (Yoh *et al*, 2007; Brès *et al*, 2008). Therefore, the potential role of Supt6h or other chaperones in resetting of mammalian chromatin remains an open question.

Chapter Six - Final discussion and future directions

The signalling events and transcription factors controlling activation of the *GM-CSF* gene in T cells have been well characterised, however, little was known regarding epigenetic control of the gene at the commencement of this thesis. The data presented in this thesis build upon prior knowledge to expand our understanding of the factors that determine the response of the *GM-CSF* gene to activating signals. Specifically, key differences were observed between T cells, in which *GM-CSF* expression can be induced, and B cells, in which it cannot. This difference in inducibility between T and B cells was seen in both transformed cell lines and primary cells.

A model of regulation of the *GM-CSF* gene as it was understood prior to the work presented here was shown in Figure 1.2. In unstimulated cells, a nucleosome covers the proximal promoter. The Brg1 chromatin remodelling protein is enriched at the *GM-CSF* promoter in EL-4 T cells relative to A20 B cells (Brettingham-Moore *et al*, 2008). Upon stimulation, Rel/NF-κB proteins translocate to the nucleus, the promoter nucleosome is remodelled, allowing assembly of the transcription complex, and *GM-CSF* mRNA transcription takes place (Brettingham-Moore *et al*, 2005). In A20 B cells, this promoter remodelling does not take place and transcription is not initiated.

This model can now be expanded upon (Figure 6.1). In EL-4 T cells the *GM-CSF* promoter is marked by increased levels of histone H3 acetylation and Sp1 binding, while in A20 B cells the promoter has increased levels of DNA methylation and histone H3 lysine 27 trimethylation. These modifications create a repressive environment at the *GM-CSF* promoter in A20 B cells that is unresponsive to stimulation, while in T cells the *GM-CSF* promoter environment is permissive, allowing chromatin remodelling and gene expression in response to stimulation. In primary $CD4^+$ T cells and $CD19^+$ B cells the *GM-CSF* promoter shares some of these features with the respective cell lines, with T cells displaying higher levels of H3 acetylation and Brg1 enrichment at the *GM-CSF* promoter than B cells. However, there was no difference in DNA methylation status of the *GM-CSF* promoter in primary T compared to B cells. Thus, it should be noted that the model in Fig. 6.1 does not apply to primary cells, but rather transformed cells only.

Epigenetic marks were found to play a role in regulating expression of the *GM-CSF* gene, as in A20 B cells a permissive chromatin environment could be generated at the *GM-CSF* promoter by treatment with the HDAC inhibitor Trichostatin A, which increases H3 acetylation at the promoter. Treatment with 2-deoxy-5-azacytidine decreased DNA methylation at the promoter, but was not sufficient to facilitate GM-CSF gene transcription in A20 B cells. However, azacytidine treatment had a synergistic effect on *GM-CSF* transcription when used in concert with TSA treatment.

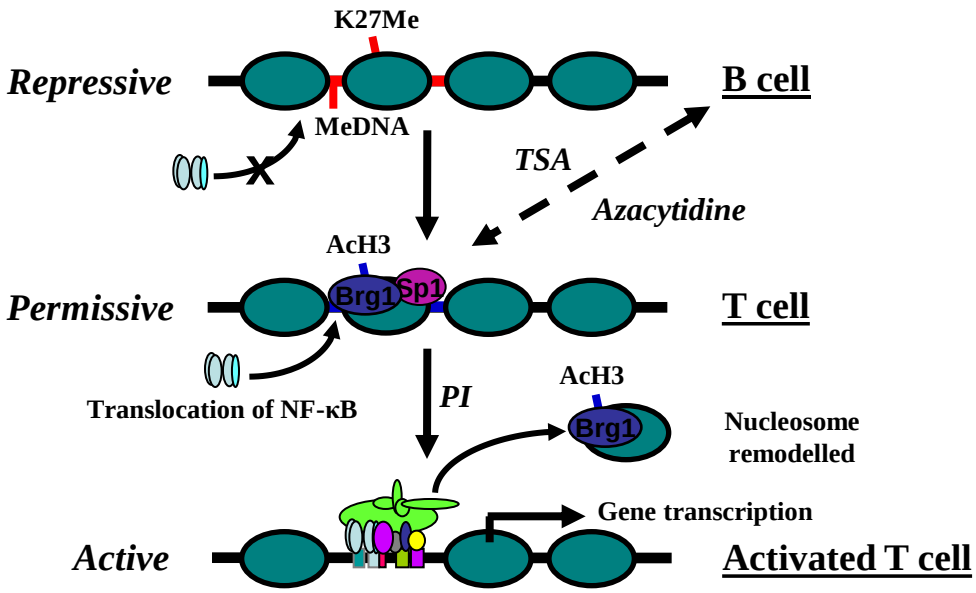

Figure 6.1: Model of *GM-CSF* regulation in transformed T and B cell lines. In A20 B cells, the *GM-CSF* promoter is marked by relatively low levels of histone H3 acetylation, Brg1 association and Sp1 binding, and relatively high levels of H3 lysine 27 trimethylation and DNA methylation, creating an environment repressive (red) to gene activation. In EL-4 T cells, the opposite situation is present, creating a permissive (blue) state. Upon PI stimulation, T cells are able to respond to NF-κB signalling and undergo promoter remodelling and transcription of *GM-CSF*, while B cells do not. A permissive state can be induced in B cells by treatment with TSA and azacytidine, which increase H3 acetylation and lower DNA methylation respectively; however, this state is not maintained following resetting of the chromatin, and the gene reverts to an repressive state.

There are several potential mechanisms by which these epigenetic marks may regulate inducibility of the *GM-CSF* gene. The SWI/SNF chromatin remodelling complex has been implicated in activation of the *GM-CSF* gene in response to T cell signalling. In Brettingham-Moore *et al* (2008), EL-4 T cells were transfected with a mutant construct of the SWI/SNF core component Brg1 containing an inactive ATPase domain. Expression of this mutant Brg1 protein resulted in delayed chromatin remodelling at the *GM-CSF* promoter and reduced levels of *GM-CSF* mRNA transcription. This suggests that Brg1 is required for rapid remodelling of the *GM-CSF* promoter and subsequent gene transcription. Two of the factors examined in this thesis may play a role in recruiting Brg1 to the *GM-CSF* promoter in unstimulated EL-4 T cells. The Brg1 protein contains a bromodomain, which binds to acetylated lysine residues on histone H3 *in vitro* (Shen *et al*, 2007; Singh *et al*, 2007). Furthermore, a mutant form of the yeast Brg1 homolog Swi2/Snf2 with the bromodomain deleted is unable to bind acetylated nucleosomes effectively, and exhibits reduced remodelling activity compared to the wild type (Awad and Hassan, 2008). Since treatment with TSA increases histone H3 acetylation at the *GM-CSF* promoter, and allows chromatin remodelling and gene transcription, it is possible that Brg1 is recruited to the promoter by interactions with hyperacetylated histones at the *GM-CSF* promoter. TSA treatment has previously been observed to result in increased Brg1 recruitment to the *GM-CSF* promoter in EL-4 T cells (Brettingham-Moore *et al*, 2008), and a trend towards increased Brg1 recruitment at the *GM-CSF*

promoter in A20 B cells in response to TSA treatment was observed during this thesis, but the data was inconclusive (data not shown).

Another factor that may be involved in tethering Brg1 to the *GM-CSF* promoter could be the Sp1 transcription factor. Sp1 is enriched at the promoter in EL-4 T cells compared to A20 B cells, and previous studies have demonstrated interactions between Sp1 and Brg1. Ma *et al* (2004) demonstrated interactions between Brg1 and Sp1 at the promoter of the human matrix metalloproteinase-2 (*MMP2*) gene. Liu *et al* (2002) found that Sp1 and the BAF complex are constitutively associated with the interferon-induced transmembrane protein 3 (*IFITM3*) gene promoter, with mutation of the Sp1 binding site causing a reduction in IFN-α induced and BAF-mediated *IFITM3* gene expression. This constitutive association of Sp1 and the BAF complex with the gene promoter is similar to the situation observed for the *GM-CSF* gene. Therefore, histone H3 acetylation and/or Sp1 binding may mediate recruitment of Brg1 to the *GM-CSF* promoter. Future work could focus on confirming if increasing H3 acetylation at the *GM-CSF* promoter with TSA increases Brg1 recruitment, and a Brg1 bromodomain mutant plasmid construct such as that previously published in Inayoshi *et al* (2006) could be used to further examine the role of the Brg1 bromodomain in recruitment of the chromatin remodelling complex to the *GM-CSF* promoter. In the case of Sp1, chromatin immunoprecipitation could be used to determine the binding status of Brg1 to the promoter of the stably transfected Sp1m *GM-CSF* construct examined in Chapter 3. Additionally, it has recently been

suggested that a RUNX1 binding site in the *GM-CSF* promoter may play a role in recruiting Brg1 to the *GM-CSF* promoter (Bakshi *et al*, 2010).

Sp1 may also be a factor in regulation of the *GM-CSF* promoter by DNA methylation. The Sp1 binding site contains a CpG dinucleotide, and several studies have shown that methylation of the Sp1 binding site in other promoters hinders Sp1 binding (Zhang *et al*, 2007; Iacobazzi *et al*, 2008). Therefore, increased methylation of the Sp1 binding site in A20 B cells may explain the decreased Sp1 recruitment to the *GM-CSF* promoter observed in these cells. Demethylation of the promoter with azacytidine or methylation of a promoter construct with *Sss*I methylase could be used to examine the effect of DNA methylation on Sp1 binding to the *GM-CSF* promoter.

DNA methylation has also been associated with H3K27 trimethylation, and both of these marks are generally associated with gene repression. In A20 B cells, higher levels of H3K27me3 (and DNA methylation) were seen at the *GM-CSF* promoter than in EL4 T cells. Studies suggest that H3K27 methylation may play a precursor role to DNA methylation in gene repression. The H3K27me3 mark is established by EZH2, a member of the PRC2 complex, which is in turn recruited by H3K27me3 (Hansen and Helin, 2009). Vire *et al* (2006) demonstrated that EZH2 associates with DNA methyltransferases both *in vitro* and *in vivo*, as well as facilitating DNA methylation of EZH2 target promoters. Histone and DNA methylation were also shown to interact to regulate the *RUNX3* gene. Knockdown of EZH2 resulted in loss of H3K27me3 from the *RUNX3* promoter in colorectal cancer cell lines, but did not

cause increased *RUNX3* expression. However, when *RUNX3* gene expression was facilitated by treatment with TSA and azacytidine, subsequent knockdown of EZH2 prevented the re-establishment of *RUNX3* silencing (Kodach *et al*, 2010). Therefore, it was suggested that H3K27 methylation occurs prior to DNA methylation, resulting in the recruitment of DNMTs and establishment of more permanent gene silencing via DNA methylation. As azacytidine and TSA were found to facilitate *GM-CSF* gene expression in A20 B cells, a similar experiment to that performed by Kodach could be performed to examine the contribution of EZH2 and H3K27me3 to the establishment of DNA methylation in the A20 B cell line.

H3K27me3, or other Polycomb-mediated histone modifications, may also contribute to *GM-CSF* gene repression in primary B cells. No differences in DNA methylation at the *GM-CSF* promoter were observed between primary T and B cells. It has been theorised that in primary cells, gene silencing may be regulated by Polycomb mechanisms, with an epigenetic switch to DNA methylation-governed silencing taking place in transformed cells (Gal-Yam *et al*, 2008). Similar switching may occur between primary B and A20 B cells, and thus investigation of repressive histone methylation marks at the *GM-CSF* promoter in primary cells may be a fruitful area of future study.

While TSA treatment was able to facilitate *GM-CSF* expression in A20 B cells in response to subsequent stimulation, the permissive chromatin state induced by TSA was not maintained following resetting of the promoter chromatin and transcriptional

downregulation of the *GM-CSF* gene. Specifically, the elevated AcH3 levels at the *GM-CSF* promoter generated by TSA treatment did not persist following reassembly of the promoter nucleosome. TSA treatment allowed nucleosomal remodelling at the *GM-CSF* promoter upon stimulation, with reassembly of the chromatin taking place upon stimulus withdrawal. The reassembled chromatin did not maintain the hyperacetylated histone H3 state induced by TSA, and was unresponsive to subsequent stimulation. The reassembly of chromatin at gene promoters has been examined at yeast genes, most notably *PHO5* (Adkins *et al*, 2004; Adkins *et al*, 2006); however, this work has mainly focused on the kinetics of promoter chromatin reassembly and involvement of histone chaperones in the process. As a result the maintenance of histone modifications during promoter chromatin resetting is not well understood. Although the mechanisms of chromatin reassembly at the *GM-CSF* promoter remain unclear, transcriptional downregulation of the *GM-CSF* gene in A20 B cells was found to be independent of protein synthesis. Further research is required to determine whether new histones are deposited at the promoter during cell division, or by a mechanism independent of DNA replication. While TSA treatment enabled *GM-CSF* gene transcription in A20 B cells by increasing promoter AcH3 levels, if repressive DNA and histone methylation marks remain, they may recruit HDACs and other repressive factors to the *GM-CSF* promoter that mediate the return of the chromatin to an unresponsive state. Association of HDACs with proteins that bind methylated DNA and methylated histones has been observed at inactive gene promoters previously (Matarazzo *et al*, 2007). The distribution of these factors at the

GM-CSF promoter in A20 B cells could be studied during activation and subsequent downregulation of the gene.

Overall, the experiments undertaken in this thesis have expanded what is known about epigenetic control of inducible *GM-CSF* gene expression, in both permissive and repressive environments within the immune system. In recent years, advances in technology have allowed genome-wide studies to be undertaken to examine the distribution of epigenetic marks on the promoters of inducible and repressed genes in immune cells, as seen in such studies as Lim *et al* (2009) and Ramirez-Carrozzi *et al* (2009). These studies have identified broad principles about epigenetic mechanisms of inducible cytokine regulation, which are complemented by single gene studies such as the one reported here. For example, Lim *et al* (2009) reported enrichment of histone acetylation and reduced H3K27me3 levels at highly inducible gene promoters. This pattern was also observed at the inducible EL-4 T cell *GM-CSF* promoter in this study. Ramirez-Carrozzi *et al* (2009) observed that inducible gene promoters lacking CpG islands tend to assemble into stable nucleosomes and require the SWI/SNF complex for inducible gene expression, in keeping with the data presented here for *GM-CSF*. Future work on the *GM-CSF* promoter, building on the results reported in this thesis, is likely to provide insight into the general mechanisms by which these observed epigenetic differences repress or permit inducible gene transcription.

References

Abbas, A. K., K. M. Murphy and A. Sher (1996). Functional diversity of helper T lymphocytes. Nature 383(6603): 787-93.

Adkins, M. W., S. R. Howar and J. K. Tyler (2004). Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. Mol Cell 14(5): 657-66.

Adkins, M. W. and J. K. Tyler (2006). Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. Mol Cell 21(3): 405-16.

Adkins, M. W., S. K. Williams, J. Linger and J. K. Tyler (2007). Chromatin disassembly from the PHO5 promoter is essential for the recruitment of the general transcription machinery and coactivators. Mol Cell Biol 27(18): 6372-82.

Agalioti, T., S. Lomvardas, B. Parekh, J. Yie, T. Maniatis and D. Thanos (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell 103(4): 667-78.

Agalioti, T., G. Chen and D. Thanos (2002). Deciphering the transcriptional histone acetylation code for a human gene. Cell 111(3): 381-92.

Agarwal, S. and A. Rao (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 9(6): 765-75.

Agarwal, S., O. Avni and A. Rao (2000). Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer in vivo. Immunity 12(6): 643-52.

Angkasekwinai, P., H. Park, Y. H. Wang, S. H. Chang, D. B. Corry, Y. J. Liu, Z. Zhu and C. Dong (2007). Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med 204(7): 1509-17.

Arya, G., A. Maitra and S. A. Grigoryev (2010). A structural perspective on the where, how, why, and what of nucleosome positioning. J Biomol Struct Dyn 27(6): 803-20.

Aune, T. M., P. L. Collins and S. Chang (2009). Epigenetics and T helper 1 differentiation. Immunology 126(3): 299-305.

Awad, S. and A. H. Hassan (2008). The Swi2/Snf2 bromodomain is important for the full binding and remodeling activity of the SWI/SNF complex on H3- and H4-acetylated nucleosomes. Ann N Y Acad Sci 1138: 366-75.

Bakshi, R., M. Q. Hassan, J. Pratap, J. B. Lian, M. A. Montecino, A. J. van Wijnen, J. L. Stein, A. N. Imbalzano and G. S. Stein (2010). The human SWI/SNF complex associates with RUNX1 to control transcription of hematopoietic target genes. J Cell Physiol. EPub.

Barski, A., S. Cuddapah, K. Cui, T. Y. Roh, D. E. Schones, Z. Wang, G. Wei, I. Chepelev and K. Zhao (2007). High-resolution profiling of histone methylations in the human genome. Cell 129(4): 823-37.

Bell, A. C. and G. Felsenfeld (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405(6785): 482-5.

Berger, S. L. (2007). The complex language of chromatin regulation during transcription. Nature 447(7143): 407-12.

Bestor, T. H. (2000). The DNA methyltransferases of mammals. Hum Mol Genet 9(16): 2395-402.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev 16(1): 6-21.

Bird, A. (2007). Perceptions of epigenetics. Nature 447(7143): 396-8.

Boeger, H., J. Griesenbeck, J. S. Strattan and R. D. Kornberg (2003). Nucleosomes unfold completely at a transcriptionally active promoter. Mol Cell 11(6): 1587-98.

Bogdanovic, O. and G. J. Veenstra (2009). DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma 118(5): 549-65.

Brandeis, M., D. Frank, I. Keshet, Z. Siegfried, M. Mendelsohn, A. Nemes, V. Temper, A. Razin and H. Cedar (1994). Sp1 elements protect a CpG island from de novo methylation. Nature 371(6496): 435-8.

Brehm, A., K. R. Tufteland, R. Aasland and P. B. Becker (2004). The many colours of chromodomains. Bioessays 26(2): 133-40.

Bres, V., S. M. Yoh and K. A. Jones (2008). The multi-tasking P-TEFb complex. Curr Opin Cell Biol 20(3): 334-40.

Bretscher, P. A. (1999). A two-step, two-signal model for the primary activation of precursor helper T cells. Proc Natl Acad Sci U S A 96(1): 185-90.

Brettingham-Moore, K. H., S. Rao, T. Juelich, M. F. Shannon and A. F. Holloway (2005). GM-CSF promoter chromatin remodelling and gene transcription display

distinct signal and transcription factor requirements. Nucleic Acids Res 33(1): 225-34.

Brettingham-Moore, K. H., O. R. Sprod, X. Chen, P. Oakford, M. F. Shannon and A. F. Holloway (2008). Determinants of a transcriptionally competent environment at the GM-CSF promoter. Nucleic Acids Res 36(8): 2639-53.

Bruniquel, D. and R. H. Schwartz (2003). Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol 4(3): 235-40.

Caamano, J. and C. A. Hunter (2002). NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. Clin Microbiol Rev 15(3): 414-29.

Cakouros, D., P. N. Cockerill, A. G. Bert, R. Mital, D. C. Roberts and M. F. Shannon (2001). A NF-kappa B/Sp1 region is essential for chromatin remodeling and correct transcription of a human granulocyte-macrophage colony-stimulating factor transgene. J Immunol 167(1): 302-10.

Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones and Y. Zhang (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 298(5595): 1039-43.

Carey, M. (1998). The enhanceosome and transcriptional synergy. Cell 92(1): 5-8.

Chahrour, M., S. Y. Jung, C. Shaw, X. Zhou, S. T. Wong, J. Qin and H. Y. Zoghbi (2008). MeCP2, a key contributor to neurological disease, activates and represses transcription. Science 320(5880): 1224-9.

Chandy, M., J. L. Gutierrez, P. Prochasson and J. L. Workman (2006). SWI/SNF displaces SAGA-acetylated nucleosomes. Eukaryot Cell 5(10): 1738-47.

Chang, H. D., C. Helbig, L. Tykocinski, S. Kreher, J. Koeck, U. Niesner and A. Radbruch (2007). Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. Eur J Immunol 37(3): 807-17.

Chaplin, D. D. (2010). Overview of the immune response. J Allergy Clin Immunol 125(2 Suppl 2): S3-23.

Chen, X., J. Wang, D. Woltring, S. Gerondakis and M. F. Shannon (2005). Histone dynamics on the interleukin-2 gene in response to T-cell activation. Mol Cell Biol 25(8): 3209-19.

Chiang, P. W., S. Wang, P. Smithivas, W. J. Song, S. Ramamoorthy, J. Hillman, S. Puett, M. L. Van Keuren, E. Crombez, A. Kumar, T. W. Glover, D. E. Miller, C. H. Tsai, C. C. Blackburn, X. N. Chen, Z. Sun, J. F. Cheng, J. R. Korenberg and D. M. Kurnit (1996). Identification and analysis of the human and murine putative chromatin structure regulator SUPT6H and Supt6h. Genomics 34(3): 328-33.

Choi, J. K. and L. J. Howe (2009). Histone acetylation: truth of consequences? Biochem Cell Biol 87(1): 139-50.

Clark, S. J. and J. Melki (2002). DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 21(35): 5380-7.

Cockerill, P. N., M. F. Shannon, A. G. Bert, G. R. Ryan and M. A. Vadas (1993). The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proc Natl Acad Sci U S A 90(6): 2466-70.

Cockerill, G. W., A. G. Bert, G. R. Ryan, J. R. Gamble, M. A. Vadas and P. N. Cockerill (1995). Regulation of granulocyte-macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT. Blood 86(7): 2689-98.

Cockerill, P. N., A. G. Bert, D. Roberts and M. A. Vadas (1999). The human granulocyte-macrophage colony-stimulating factor gene is autonomously regulated in vivo by an inducible tissue-specific enhancer. Proc Natl Acad Sci U S A 96(26): 15097-102.

Cockerill, P. N. (2004). Mechanisms of transcriptional regulation of the human IL-3/GM-CSF locus by inducible tissue-specific promoters and enhancers. Crit Rev Immunol 24(6): 385-408.

Dace, D. S., A. A. Khan, J. L. Stark, J. Kelly, A. H. Cross and R. S. Apte (2009). Interleukin-10 overexpression promotes Fas-ligand-dependent chronic macrophage-mediated demyelinating polyneuropathy. PLoS One 4(9): e7121.

de Gorter, D. J., J. C. Vos, S. T. Pals and M. Spaargaren (2007). The B cell antigen receptor controls AP-1 and NFAT activity through Ras-mediated activation of Ral. J Immunol 178(3): 1405-14.

de la Cruz, X., S. Lois, S. Sanchez-Molina and M. A. Martinez-Balbas (2005). Do protein motifs read the histone code? Bioessays 27(2): 164-75.

Donati, G., C. Imbriano and R. Mantovani (2006). Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters. Nucleic Acids Res 34(10): 3116-27.

Dunn, S. M., L. S. Coles, R. K. Lang, S. Gerondakis, M. A. Vadas and M. F. Shannon (1994). Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. Blood 83(9): 2469-79.

Dupont, C., D. R. Armant and C. A. Brenner (2009). Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med 27(5): 351-7.

Fatemi, M. and P. A. Wade (2006). MBD family proteins: reading the epigenetic code. J Cell Sci 119(Pt 15): 3033-7.

Field, L. M., F. Lyko, M. Mandrioli and G. Prantera (2004). DNA methylation in insects. Insect Mol Biol 13(2): 109-15.

Fields, P. E., S. T. Kim and R. A. Flavell (2002). Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. J Immunol 169(2): 647-50.

Fuks, F., P. J. Hurd, D. Wolf, X. Nan, A. P. Bird and T. Kouzarides (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278(6): 4035-40.

Gal-Yam, E. N., G. Egger, L. Iniguez, H. Holster, S. Einarsson, X. Zhang, J. C. Lin, G. Liang, P. A. Jones and A. Tanay (2008). Frequent switching of Polycomb repressive marks and DNA hypermethylation in the PC3 prostate cancer cell line. Proc Natl Acad Sci U S A 105(35): 12979-84.

Gangaraju, V. K. and B. Bartholomew (2007). Mechanisms of ATP dependent chromatin remodeling. Mutat Res 618(1-2): 3-17.

Gao, H., K. Lukin, J. Ramirez, S. Fields, D. Lopez and J. Hagman (2009). Opposing effects of SWI/SNF and Mi-2/NuRD chromatin remodeling complexes on epigenetic reprogramming by EBF and Pax5. Proc Natl Acad Sci U S A 106(27): 11258-63.

Gasson, J. C. (1991). Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood 77(6): 1131-45.

Gauld, S. B. and J. C. Cambier (2004). Src-family kinases in B-cell development and signaling. Oncogene 23(48): 8001-6.

Gilmore, T. D. (1999). The Rel/NF-kappaB signal transduction pathway: introduction. Oncogene 18(49): 6842-4.

Gkikopoulos, T., K. M. Havas, H. Dewar and T. Owen-Hughes (2009). SWI/SNF and Asf1p cooperate to displace histones during induction of the saccharomyces cerevisiae HO promoter. Mol Cell Biol 29(15): 4057-66.

Gutierrez, J. L., M. Chandy, M. J. Carrozza and J. L. Workman (2007). Activation domains drive nucleosome eviction by SWI/SNF. EMBO J 26(3): 730-40.

Gutschalk, C. M., C. C. Herold-Mende, N. E. Fusenig and M. M. Mueller (2006). Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor promote malignant growth of cells from head and neck squamous cell carcinomas in vivo. Cancer Res 66(16): 8026-36.

Hansen, K. H. and K. Helin (2009). Epigenetic inheritance through self-recruitment of the polycomb repressive complex 2. Epigenetics 4(3): 133-8.

Harwood, N. E. and F. D. Batista (2008). New insights into the early molecular events underlying B cell activation. Immunity 28(5): 609-19.

Hassan, A. H., P. Prochasson, K. E. Neely, S. C. Galasinski, M. Chandy, M. J. Carrozza and J. L. Workman (2002). Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. Cell 111(3): 369-79.

Henderson, I. R. and S. E. Jacobsen (2007). Epigenetic inheritance in plants. Nature 447(7143): 418-24.

Hildmann, C., D. Riester and A. Schwienhorst (2007). Histone deacetylases--an important class of cellular regulators with a variety of functions. Appl Microbiol Biotechnol 75(3): 487-97.

Himes, S. R., R. Katsikeros and M. F. Shannon (1996). Costimulation of cytokine gene expression in T cells by the human T leukemia/lymphotropic virus type 1 trans activator Tax. J Virol 70(6): 4001-8.

Holloway, A. F., S. Rao, X. Chen and M. F. Shannon (2003). Changes in chromatin accessibility across the GM-CSF promoter upon T cell activation are dependent on nuclear factor kappaB proteins. J Exp Med 197(4): 413-23.

Huang, J., P. F. Lo, T. Zal, N. R. Gascoigne, B. A. Smith, S. D. Levin and H. M. Grey (2002). CD28 plays a critical role in the segregation of PKC theta within the immunologic synapse. Proc Natl Acad Sci U S A 99(14): 9369-73.

Iacobazzi, V., V. Infantino and F. Palmieri (2008). Epigenetic mechanisms and Sp1 regulate mitochondrial citrate carrier gene expression. Biochem Biophys Res Commun 376(1): 15-20.

Illingworth, R. S. and A. P. Bird (2009). CpG islands--'a rough guide'. FEBS Lett 583(11): 1713-20.

Inayoshi, Y., K. Miyake, Y. Machida, H. Kaneoka, M. Terajima, T. Dohda, M. Takahashi and S. Iijima (2006). Mammalian chromatin remodeling complex SWI/SNF is essential for enhanced expression of the albumin gene during liver development. J Biochem 139(2): 177-88.

Jaenisch, R. and A. Bird (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl: 245-54.

Jenkins, F., P. N. Cockerill, D. Bohmann and M. F. Shannon (1995). Multiple signals are required for function of the human granulocyte-macrophage colony-stimulating factor gene promoter in T cells. J Immunol 155(3): 1240-51.

Jensen, M. M., M. S. Christensen, B. Bonven and T. H. Jensen (2008). Requirements for chromatin reassembly during transcriptional downregulation of a heat shock gene in Saccharomyces cerevisiae. FEBS J 275(11): 2956-64.

Jeong, S. and A. Stein (1994). Micrococcal nuclease digestion of nuclei reveals extended nucleosome ladders having anomalous DNA lengths for chromatin assembled on non-replicating plasmids in transfected cells. Nucleic Acids Res 22(3): 370-5.

Jones, P. A. and S. B. Baylin (2007). The epigenomics of cancer. Cell 128(4): 683-92.

Kahn-Perles, B., C. Lipcey, P. Lecine, D. Olive and J. Imbert (1997). Temporal and subunit-specific modulations of the Rel/NF-kappaB transcription factors through CD28 costimulation. J Biol Chem 272(35): 21774-83.

Kane, L. P. and A. Weiss (2003). The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. Immunol Rev 192: 7-20.

Kaushansky, K., C. O'Rork, S. G. Shoemaker and J. McCarty (1996). The regulation of GM-CSF is dependent on a complex interplay of multiple nuclear proteins. Mol Immunol 33(4-5): 461-70.

Kiani, A., F. J. Garcia-Cozar, I. Habermann, S. Laforsch, T. Aebischer, G. Ehninger and A. Rao (2001). Regulation of interferon-gamma gene expression by nuclear factor of activated T cells. Blood 98(5): 1480-8.

Kim, S. T., P. E. Fields and R. A. Flavell (2007). Demethylation of a specific hypersensitive site in the Th2 locus control region. Proc Natl Acad Sci U S A 104(43): 17052-7.

Kimura, A., K. Matsubara and M. Horikoshi (2005). A decade of histone acetylation: marking eukaryotic chromosomes with specific codes. J Biochem 138(6): 647-62.

Kitazawa, S. and R. Kitazawa (2002). Epigenetic control of mouse receptor activator of NF-kappa B ligand gene expression. Biochem Biophys Res Commun 293(1): 126-31.

Kochetkova, M., P. O. Iversen, A. F. Lopez and M. F. Shannon (1997). Deoxyribonucleic acid triplex formation inhibits granulocyte macrophage colony-stimulating factor gene expression and suppresses growth in juvenile myelomonocytic leukemic cells. J Clin Invest 99(12): 3000-8.

Kodach, L. L., R. J. Jacobs, J. Heijmans, C. J. van Noesel, A. M. Langers, H. W. Verspaget, D. W. Hommes, G. J. Offerhaus, G. R. van den Brink and J. C. Hardwick (2010). The role of EZH2 and DNA methylation in the silencing of the tumour suppressor RUNX3 in colorectal cancer. Carcinogenesis. EPub.

Kornberg, R. D. and Y. Lorch (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell 98(3): 285-94.

Kulaeva, O. I., D. A. Gaykalova and V. M. Studitsky (2007). Transcription through chromatin by RNA polymerase II: histone displacement and exchange. Mutat Res 618(1-2): 116-29.

Kusaba, H., M. Nakayama, T. Harada, M. Nomoto, K. Kohno, M. Kuwano and M. Wada (1999). Association of 5' CpG demethylation and altered chromatin structure in the promoter region with transcriptional activation of the multidrug resistance 1 gene in human cancer cells. Eur J Biochem 262(3): 924-32.

Lai, J. H., G. Horvath, J. Subleski, J. Bruder, P. Ghosh and T. H. Tan (1995). RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter. Mol Cell Biol 15(8): 4260-71.

Langst, G. and P. B. Becker (2001). Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. J Cell Sci 114(Pt 14): 2561-8.

Lee, D. U., S. Agarwal and A. Rao (2002). Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. Immunity 16(5): 649-60.

Li, B., C. C. Adams and J. L. Workman (1994). Nucleosome binding by the constitutive transcription factor Sp1. J Biol Chem 269(10): 7756-63.

Li, L., S. He, J. M. Sun and J. R. Davie (2004). Gene regulation by Sp1 and Sp3. Biochem Cell Biol 82(4): 460-71.

Lim, P. S., K. Hardy, K. L. Bunting, L. Ma, K. Peng, X. Chen and M. F. Shannon (2009). Defining the chromatin signature of inducible genes in T cells. Genome Biol 10(10): R107.

Liu, H., H. Kang, R. Liu, X. Chen and K. Zhao (2002). Maximal induction of a subset of interferon target genes requires the chromatin-remodeling activity of the BAF complex. Mol Cell Biol 22(18): 6471-9.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389(6648): 251-60.

Luo, C., E. Burgeon, J. A. Carew, P. G. McCaffrey, T. M. Badalian, W. S. Lane, P. G. Hogan and A. Rao (1996). Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Mol Cell Biol 16(7): 3955-66.

Ma, Z., M. J. Chang, R. Shah, J. Adamski, X. Zhao and E. N. Benveniste (2004). Brg-1 is required for maximal transcription of the human matrix metalloproteinase-2 gene. J Biol Chem 279(44): 46326-34.

Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5(6): 472-84.

Macleod, D., J. Charlton, J. Mullins and A. P. Bird (1994). Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev 8(19): 2282-92.

Martinez-Moczygemba, M. and D. P. Huston (2003). Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. J Allergy Clin Immunol 112(4): 653-65.

Masuda, E. S., Y. Yamaguchi-Iwai, A. Tsuboi, P. Hung, K. Arai and N. Arai (1994). The transcription factor Sp1 is required for induction of the murine GM-CSF promoter in T cells. Biochem Biophys Res Commun 205(3): 1518-25.

Matarazzo, M. R., M. L. De Bonis, M. Strazzullo, A. Cerase, M. Ferraro, P. Vastarelli, E. Ballestar, M. Esteller, S. Kudo and M. D'Esposito (2007). Multiple binding of methyl-CpG and polycomb proteins in long-term gene silencing events. J Cell Physiol 210(3): 711-9.

Medrano, E. E. and A. B. Pardee (1980). Prevalent deficiency in tumor cells of cycloheximide-induced cycle arrest. Proc Natl Acad Sci U S A 77(7): 4123-6.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. Nature 449(7164): 819-26.

Meehan, R. R., J. D. Lewis and A. P. Bird (1992). Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acids Res 20(19): 5085-92.

Mizzen, C. A. and C. D. Allis (1998). Linking histone acetylation to transcriptional regulation. Cell Mol Life Sci 54(1): 6-20.

Mondino, A. and M. K. Jenkins (1994). Surface proteins involved in T cell costimulation. J Leukoc Biol 55(6): 805-15.

Moreira, J. M., P. Scheipers and P. Sorensen (2003). The histone deacetylase inhibitor Trichostatin A modulates CD4+ T cell responses. BMC Cancer 3: 30.

Morse, R. H. (2007). Transcription factor access to promoter elements. J Cell Biochem 102(3): 560-70.

Mosmann, T. R. and R. L. Coffman (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7: 145-73.

Murrell, A., V. K. Rakyan and S. Beck (2005). From genome to epigenome. Hum Mol Genet 14 Spec No 1: R3-R10.

Nan, X., H. H. Ng, C. A. Johnson, C. D. Laherty, B. M. Turner, R. N. Eisenman and A. Bird (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393(6683): 386-9.

Nathan, C. (2002). Points of control in inflammation. Nature 420(6917): 846-52.

Nie, Z., Y. Xue, D. Yang, S. Zhou, B. J. Deroo, T. K. Archer and W. Wang (2000). A specificity and targeting subunit of a human SWI/SNF family-related chromatin-remodeling complex. Mol Cell Biol 20(23): 8879-88.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity 8(3): 275-83.

Oh, J., D. H. Sohn, M. Ko, H. Chung, S. H. Jeon and R. H. Seong (2008). BAF60a interacts with p53 to recruit the SWI/SNF complex. J Biol Chem 283(18): 11924-34.

Ohlsson, R. and C. Kanduri (2002). New twists on the epigenetics of CpG islands. Genome Res 12(4): 525-6.

Ohsawa, R., M. Adkins and J. K. Tyler (2009). Epigenetic inheritance of an inducibly nucleosome-depleted promoter and its associated transcriptional state in the apparent absence of transcriptional activators. Epigenetics Chromatin 2(1): 11.

Okano, M., D. W. Bell, D. A. Haber and E. Li (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99(3): 247-57.

Osborne, C. S., M. A. Vadas and P. N. Cockerill (1995). Transcriptional regulation of mouse granulocyte-macrophage colony-stimulating factor/IL-3 locus. J Immunol 155(1): 226-35.

Pazin, M. J., P. L. Sheridan, K. Cannon, Z. Cao, J. G. Keck, J. T. Kadonaga and K. A. Jones (1996). NF-kappa B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. Genes Dev 10(1): 37-49.

Ramirez-Carrozzi, V. R., A. A. Nazarian, C. C. Li, S. L. Gore, R. Sridharan, A. N. Imbalzano and S. T. Smale (2006). Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. Genes Dev 20(3): 282-96.

Ramirez-Carrozzi, V. R., D. Braas, D. M. Bhatt, C. S. Cheng, C. Hong, K. R. Doty, J. C. Black, A. Hoffmann, M. Carey and S. T. Smale (2009). A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell 138(1): 114-28.

Ransom, M., B. K. Dennehey and J. K. Tyler (2010). Chaperoning histones during DNA replication and repair. Cell 140(2): 183-95.

Rao, S., E. Procko and M. F. Shannon (2001). Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. J Immunol 167(8): 4494-503.

Rea, S., F. Eisenhaber, D. O'Carroll, B. D. Strahl, Z. W. Sun, M. Schmid, S. Opravil, K. Mechtler, C. P. Ponting, C. D. Allis and T. Jenuwein (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406(6796): 593-9.

Reinke, H., P. D. Gregory and W. Horz (2001). A transient histone hyperacetylation signal marks nucleosomes for remodeling at the PHO8 promoter in vivo. Mol Cell 7(3): 529-38.

Reinke, H. and W. Horz (2003). Histones are first hyperacetylated and then lose contact with the activated PHO5 promoter. Mol Cell 11(6): 1599-607.

Roh, T. Y., S. Cuddapah and K. Zhao (2005). Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping. Genes Dev 19(5): 542-52.

Roh, T. Y., S. Cuddapah, K. Cui and K. Zhao (2006). The genomic landscape of histone modifications in human T cells. Proc Natl Acad Sci U S A 103(43): 15782-7.

Rudd, C. E. and H. Schneider (2003). Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. Nat Rev Immunol 3(7): 544-56.

Rufiange, A., P. E. Jacques, W. Bhat, F. Robert and A. Nourani (2007). Genome-wide replication-independent histone H3 exchange occurs predominantly at promoters and implicates H3 K56 acetylation and Asf1. Mol Cell 27(3): 393-405.

Ruland, J. and T. W. Mak (2003). Transducing signals from antigen receptors to nuclear factor kappaB. Immunol Rev 193: 93-100.

Saha, S., C. Doe, V. Mistry, S. Siddiqui, D. Parker, M. Sleeman, E. S. Cohen and C. E. Brightling (2009). Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. Thorax 64(8): 671-6.

Schermer, U. J., P. Korber and W. Horz (2005). Histones are incorporated in trans during reassembly of the yeast PHO5 promoter. Mol Cell 19(2): 279-85.

Schmitz, M. L. and D. Krappmann (2006). Controlling NF-kappaB activation in T cells by costimulatory receptors. Cell Death Differ 13(5): 834-42.

Schreck, R. and P. A. Baeuerle (1990). NF-kappa B as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene. Mol Cell Biol 10(3): 1281-6.

Schreiber, E., P. Matthias, M. M. Muller and W. Schaffner (1989). Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 17(15): 6419.

Schubeler, D., D. M. MacAlpine, D. Scalzo, C. Wirbelauer, C. Kooperberg, F. van Leeuwen, D. E. Gottschling, L. P. O'Neill, B. M. Turner, J. Delrow, S. P. Bell and M. Groudine (2004). The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. Genes Dev 18(11): 1263-71.

Selvi, R. B. and T. K. Kundu (2009). Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. Biotechnol J 4(3): 375-90.

Shang, C., J. Attema, D. Cakouros, P. N. Cockerill and M. F. Shannon (1999). Nuclear factor of activated T cells contributes to the function of the CD28 response region of the granulocyte macrophage-colony stimulating factor promoter. Int Immunol 11(12): 1945-56.

Shankar, B., S. S. Kumar and K. B. Sainis (2003). Generation of reactive oxygen species and radiation response in lymphocytes and tumor cells. Radiat Res 160(4): 478-87.

Shannon, M. F., S. R. Himes and L. S. Coles (1995). GM-CSF and IL-2 share common control mechanisms in response to costimulatory signals in T cells. J Leukoc Biol 57(5): 767-73.

Shen, W., C. Xu, W. Huang, J. Zhang, J. E. Carlson, X. Tu, J. Wu and Y. Shi (2007). Solution structure of human Brg1 bromodomain and its specific binding to acetylated histone tails. Biochemistry 46(8): 2100-10.

Shinohara, H. and T. Kurosaki (2009). Comprehending the complex connection between PKCbeta, TAK1, and IKK in BCR signaling. Immunol Rev 232(1): 300-18.

Shoda, H., K. Fujio and K. Yamamoto (2007). Rheumatoid arthritis and interleukin-32. Cell Mol Life Sci 64(19-20): 2671-9.

Shogren-Knaak, M., H. Ishii, J. M. Sun, M. J. Pazin, J. R. Davie and C. L. Peterson (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 311(5762): 844-7.

Singh, M., G. M. Popowicz, M. Krajewski and T. A. Holak (2007). Structural ramification for acetyl-lysine recognition by the bromodomain of human BRG1 protein, a central ATPase of the SWI/SNF remodeling complex. Chembiochem 8(11): 1308-16.

Sobel, R. E., R. G. Cook, C. A. Perry, A. T. Annunziato and C. D. Allis (1995). Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. Proc Natl Acad Sci U S A 92(4): 1237-41.

Strahl, B. D. and C. D. Allis (2000). The language of covalent histone modifications. Nature 403(6765): 41-5.

Sulewska, A., W. Niklinska, M. Kozlowski, L. Minarowski, W. Naumnik, J. Niklinski, K. Dabrowska and L. Chyczewski (2007). DNA methylation in states of cell physiology and pathology. Folia Histochem Cytobiol 45(3): 149-58.

Sun, Z. and R. Andersson (2002). NF-kappaB activation and inhibition: a review. Shock 18(2): 99-106.

Svaren, J. and W. Horz (1997). Transcription factors vs nucleosomes: regulation of the PHO5 promoter in yeast. Trends Biochem Sci 22(3): 93-7.

Thanos, D. and T. Maniatis (1995a). Identification of the rel family members required for virus induction of the human beta interferon gene. Mol Cell Biol 15(1): 152-64.

Thanos, D. and T. Maniatis (1995b). Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell 83(7): 1091-100.

Thomas, R. S., M. J. Tymms, L. H. McKinlay, M. F. Shannon, A. Seth and I. Kola (1997). ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene 14(23): 2845-55.

Tokumitsu, H., E. S. Masuda, A. Tsuboi, K. Arai and N. Arai (1993). Purification of the 120 kDa component of the human nuclear factor of activated T cells (NF-AT): reconstitution of binding activity to the cis-acting element of the GM-CSF and IL-2 promoter with AP-1. Biochem Biophys Res Commun 196(2): 737-44.

Tremethick, D. J. (2007). Higher-order structures of chromatin: the elusive 30 nm fiber. Cell 128(4): 651-4.

Trotter, K. W. and T. K. Archer (2008). The BRG1 transcriptional coregulator. Nucl Recept Signal 6: e004.

Tse, C., T. Sera, A. P. Wolffe and J. C. Hansen (1998). Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III. Mol Cell Biol 18(8): 4629-38.

Tsuboi, A., M. Muramatsu, A. Tsutsumi, K. Arai and N. Arai (1994). Calcineurin activates transcription from the GM-CSF promoter in synergy with either protein kinase C or NF-kappa B/AP-1 in T cells. Biochem Biophys Res Commun 199(2): 1064-72.

Tu, N., Y. Hu and N. F. Mivechi (2006). Heat shock transcription factor (Hsf)-4b recruits Brg1 during the G1 phase of the cell cycle and regulates the expression of heat shock proteins. J Cell Biochem 98(6): 1528-42.

Upcher, W. (2007). Re-repression of the GM-CSF gene. Department of Biochemistry. University of Tasmania, Hobart. Thesis (BSc (Hons)).

van der Merwe, P. A. and S. J. Davis (2003). Molecular interactions mediating T cell antigen recognition. Annu Rev Immunol 21: 659-84.

van Holde, K. and J. Zlatanova (1996). What determines the folding of the chromatin fiber? Proc Natl Acad Sci U S A 93(20): 10548-55.

van Panhuys, N., G. Le Gros and M. J. McConnell (2008). Epigenetic regulation of Th2 cytokine expression in atopic diseases. Tissue Antigens 72(2): 91-7.

Vanti, M., E. Gallastegui, I. Respaldiza, A. Rodriguez-Gil, F. Gomez-Herreros, S. Jimeno-Gonzalez, A. Jordan and S. Chavez (2009). Yeast genetic analysis reveals the involvement of chromatin reassembly factors in repressing HIV-1 basal transcription. PLoS Genet 5(1): e1000339.

Venters, B. J. and B. F. Pugh (2009). How eukaryotic genes are transcribed. Crit Rev Biochem Mol Biol 44(2-3): 117-41.

Vignali, M., A. H. Hassan, K. E. Neely and J. L. Workman (2000). ATP-dependent chromatin-remodeling complexes. Mol Cell Biol 20(6): 1899-910.

Vire, E., C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J. M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit and F. Fuks (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature 439(7078): 871-4.

Wade, P. A., A. Gegonne, P. L. Jones, E. Ballestar, F. Aubry and A. P. Wolffe (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 23(1): 62-6.

Wang, C. Y., A. G. Bassuk, L. H. Boise, C. B. Thompson, R. Bravo and J. M. Leiden (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. Mol Cell Biol 14(2): 1153-9.

Wang, W., T. Chi, Y. Xue, S. Zhou, A. Kuo and G. R. Crabtree (1998). Architectural DNA binding by a high-mobility-group/kinesin-like subunit in mammalian SWI/SNF-related complexes. Proc Natl Acad Sci U S A 95(2): 492-8.

Wegel, E. and P. Shaw (2005). Gene activation and deactivation related changes in the three-dimensional structure of chromatin. Chromosoma 114(5): 331-7.

Weil, R. and A. Israel (2006). Deciphering the pathway from the TCR to NF-kappaB. Cell Death Differ 13(5): 826-33.

Weinmann, A. S., S. E. Plevy and S. T. Smale (1999). Rapid and selective remodeling of a positioned nucleosome during the induction of IL-12 p40 transcription. Immunity 11(6): 665-75.

Wen, H., Y. Dou, C. M. Hogaboam and S. L. Kunkel (2008). Epigenetic regulation of dendritic cell-derived interleukin-12 facilitates immunosuppression after a severe innate immune response. Blood 111(4): 1797-804.

West, A. G. and P. Fraser (2005). Remote control of gene transcription. Hum Mol Genet 14 Spec No 1: R101-11.

Widlak, P., R. B. Gaynor and W. T. Garrard (1997). In vitro chromatin assembly of the HIV-1 promoter. ATP-dependent polar repositioning of nucleosomes by Sp1 and NFkappaB. J Biol Chem 272(28): 17654-61.

Williams, S. K. and J. K. Tyler (2007). Transcriptional regulation by chromatin disassembly and reassembly. Curr Opin Genet Dev 17(2): 88-93.

Wohrle, S., B. Wallmen and A. Hecht (2007). Differential control of Wnt target genes involves epigenetic mechanisms and selective promoter occupancy by T-cell factors. Mol Cell Biol 27(23): 8164-77.

Workman, J. L. (2006). Nucleosome displacement in transcription. Genes Dev 20(15): 2009-17.

Xiang, Y., Z. Zhu, G. Han, H. Lin, L. Xu and C. D. Chen (2007). JMJD3 is a histone H3K27 demethylase. Cell Res 17(10): 850-7.

Yie, J., K. Senger and D. Thanos (1999). Mechanism by which the IFN-beta enhanceosome activates transcription. Proc Natl Acad Sci U S A 96(23): 13108-13.

Yoh, S. M., H. Cho, L. Pickle, R. M. Evans and K. A. Jones (2007). The Spt6 SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export. Genes Dev 21(2): 160-74.

Young, D. C., K. Wagner and J. D. Griffin (1987). Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. J Clin Invest 79(1): 100-6.

Zhang, Y. and D. Reinberg (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev 15(18): 2343-60.

Zhang, F. and M. Boothby (2006). T helper type 1-specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN-gamma promoter are Stat4 dependent. J Exp Med 203(6): 1493-505.

Zhang, X., R. Soong, K. Wang, L. Li, J. R. Davie, V. Guarcello and R. B. Diasio (2007). Suppression of DPYD expression in RKO cells via DNA methylation in the regulatory region of the DPYD promoter: a potentially important epigenetic mechanism regulating DPYD expression. Biochem Cell Biol 85(3): 337-46.

Zhu, J., F. He, S. Hu and J. Yu (2008). On the nature of human housekeeping genes. Trends Genet 24(10): 481-4.